RELATIONSHIP OF CONTROLLED ENVIRONMENTAL STRESS AND PROTEIN INTAKE TO GROWTH AND OTHER PHYSIOLOGICAL MANIFESTATIONS IN YOUNG RATS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Helen Louise Bishop
1963

ABSTRACT

RELATIONSHIP OF CONTROLLED ENVIRONMENTAL STRESS AND PROTEIN INTAKE TO GROWTH AND OTHER PHYSIOLOGICAL MANIFESTATIONS IN YOUNG RATS

by Helen Louise Bishop

The object of this experiment was to study the response of male albino rats to the stress of bright, flashing lights when the diet contained varying levels of protein. Forty rats were divided into four groups of ten rats each (Groups A, B, C, and D). Two groups (A and B) were fed a low-protein diet (9% casein) and one of these groups (B) was placed in a stressful situation by being exposed to bright, flashing lights. Two groups (C and D) received a high-protein diet (20% casein) and one of these groups (D) was also exposed to the lights.

On the 28th day, blood samples were taken from all animals for cholesterol determination and the animals were sacrificed. Portions of liver were taken for the determination of glutathione levels, and adrenal glands were removed for weighing.

In both groups subjected to the stress of the flashing lights there was a marked increase in liver glutathione levels when compared with the controls. This was interpreted as evidence that the lights were causing physiological upsets in the animals. Adrenal glands - on the basis of weight per

100 g. body weight - were found to be largest in the stressed, low-protein group and smallest in the non-stressed, high-protein group. Both high-protein groups showed highly satisfactory weight gains. The stressed, low-protein group (B) gained significantly less weight than did Group A despite the relatively similar levels of food intake in both groups, indicating that the stress might have introduced an additional requirement for protein which was met at the expense of the animals' growth. Total cholesterol levels did not vary significantly between A and B, nor between C and D. This is in accordance with the findings of other authors that a long period of time is needed for marked differences in cholesterol levels to appear in rats subjected to stress. There was no significant difference between the total cholesterol blood levels of Groups A and C - possibly because the difference in protein intake of these two groups was not extreme enough. There was, however, a difference in the total blood cholesterol level between Groups D and B, significant at the 5% level, suggesting that the combination of the stress of the flashing lights with the 11% difference in protein intake was sufficient to differentiate the cholesterolemic responses. Group B showed significantly higher levels of free serum cholesterol and Group D significantly lower levels than either Group A or Group C. It is suggested that the difference in available protein for extra cholesterol mobilization in the form of lipoprotein is the reason for this finding.

RELATIONSHIP OF CONTROLLED ENVIRONMENTAL STRESS AND PROTEIN INTAKE TO GROWTH AND OTHER PHYSIOLOGICAL MANIFESTATIONS IN YOUNG RATS

by

Helen Louise Bishop

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Foods and Nutrition

1963

ACKNOWLEDGEMENT

The author wishes to express her grateful appreciation to Dr. Theodore F. Irmiter for his advice and guidance throughout the course of this research, to the faculty and staff of the department of Foods and Nutrition for their help and encouragement, and to Michigan State University for making the research possible.

Special thanks to Sister St. Catherine Maureen, C.N.D., for her inspiration and counsel.

To My Parents

TABLE OF CONTENTS

INTRODUCTION

Ershoff (8) in a paper entitled "Nutrition and Stress", states that any factor that interferes with the digestion, absorption, or utilization of nutrients, or increases their destruction or excretion may result in malnutrition despite the apparent adequacy of the diet fed. Body requirements may furthermore be significantly increased for purposes of detoxification or by factors such as physical exertion, fever, burns, major surgical procedures, abnormal environmental conditions and others. The net result of such factors may be an increase in body requirements beyond the usual or average range and the precipitation of nutritional deficiencies on diets that would otherwise be adequate.

It would be erroneous to generalize by stating that "stress" per se increases nutritional requirements, for, as Ershoff explains, "stress" is not a specific, unvarying and distinct entity. "Stress" is a state of the organism, the result of those processes an organism employs in attempting to maintain homeostasis under unfavorable alterations in the environment. Furthermore, since life is a process in which the organism attempts to maintain itself in a changing environment, "stress" is inherent in life. Ershoff maintains that increased nutritional requirements following exposure to stressor agents result not as a consequence of "stress" per se but rather from the increased requirement for specific

nutrients due to the physiologic effect of the particular stressor agent involved. It has been demonstrated by H. Selye (35), however, that prolonged exposure to a wide variety of stressor agents results in a stereotyped response such as increased adrenaline and cholesterol levels in the stressed animals. Hence, in the present study, which employs bright, flashing lights as a stressor agent, the general usage of the term "stress" to convey the influence of the lights upon the experimental animals is thought to be a legitimate one.

Considerable data - of which Selye's are of great import - are available [Adams et al. (1), Baur and Filer (2), Denenberg and Karas (7), Ershoff (8), and Hartel (17)] indicating that diet per se and the animals nutritive state are intimately related in adaptation to stress. Varying levels of dietary protein induce biochemical changes similar to those elicited by various stressor agents [Jones and Huffman (22) and Moyer et al. (29)]. The present study explores the possibility of an association between stress and protein requirements.

Objectives

To ascertain whether:

1. Stress affects protein utilization.
2. The amount of protein in the diet has any influence on response to stress.

Hypothesis:

The effects of stress are in part a function of a protein deficiency brought about by an induced increase in protein requirements.

The recommended allowances for protein may be rendered inadequate by stressful situations.

REVIEW OF LITERATURE

A. General Effects of Stress

At about the middle of the nineteenth century the French physiologist Claude Bernard (3) had already recognized that to maintain life, the constancy of the "milieu interieur" must be preserved in spite of changes in the environment. Subsequently the American investigator Walter Cannon (5) introduced the term "homeostasis" to designate the maintenance of this steady state and directed attention to the role of adrenaline and of the autonomic nervous system in bringing about the internal adjustments upon which homeostasis depends. He listed a number of specific reactions which are important in maintaining the constancy of the "milieu interieur" during certain "emergencies" such as result from muscular work, nervous irritation and variations in the surrounding temperature.

According to Selye (35) man has had some idea, albeit subconscious, of the stress concept ever since the word "disease" has been used. The very fact that a single term can be used to denote a great variety of individual maladies, clearly indicates these maladies have something in common, possessing some nonspecific features which permit the disease state to be distinguished from the condition of health. Yet, precisely because these manifestations are not characteristic

of any one disease, they have received little attention in comparison with the specific ones. Through a series of experiments on animals, however, Ingle (21) demonstrated that the organism responds in a stereotyped manner to a variety of widely different disturbances such as: infections, intoxications, trauma, nervous strain, heat, cold or muscle fatigue. The only common feature was that they placed the body in a state of general (systemic) stress. It was concluded, therefore, that the chemical responses characteristic of all specific stresses are indicative of the general effect elicited by nonspecific systemic stress.

Selye (35) defined stress as "the sum of all nonspecific changes caused by function or damage." He reported that the first and most outstanding manifestation of this response to stress was adrenocortical enlargement.

Jurtshuk, Weltman, and Sackler (23) explored alterations in certain biochemical parameters which were measured in rats subjected to repeated, prolonged, high-intensity auditory stimulation. Specifically, levels of blood glutathione, adrenal ascorbic acid, and total adrenal cholesterol were reported. They used blood glutathione index as an indicator of "generalized" stress since Selye (35) had found that both cortisone and adrenocorticotropic hormone (ACTH) injections cause a transitory drop in the glutathione content in blood of rats and humans. The studies of Jurtshuk et al. tend to indicate that the glutathione index may be a significant

metabolic parameter in the investigation of emotional disturbances. It was the finding of the authors that severe auditory stimulation caused a marked reduction in glutathione levels in the blood of female rats. The frequency of the auditory stimulation was related inversely to the recovery rate after the stimulation. An increase in adrenal weights and adrenal ascorbic acid, as well as a decrease in total adrenal cholesterol were noted. Jurtshuk's work extended the analysis of physiologic changes reported by other workers in the field of audiogenic stress. Hurder and Sanders (19) had indicated that animals with larger adrenals were more susceptible to audiogenic seizures than animals with smaller adrenals.

Although diverse types of systemic stress have long been known to modify the immunological responses of mammals (Selye, 35) precise experimental investigations have only recently elucidated the effect of such stress on particular immune reactions. Thus, mice subjected to a standardized avoidance-learning type of stress show an increased susceptibility to herpes simplex virus infection, as well as a decreased susceptibility to passive anaphylaxis, and a depressed colloid-clearing capacity of the reticuloendothelial system (Rasmussin, 34).

Denenberg and Karas (7) explored the effects of differential infantile handling upon weight gain and mortality in the rat and mouse. Complete lack of handling or prolonged

handling both acted to reduce survival time; those animals receiving moderate handling survived a significantly longer time than either of the other two groups. The effect of infantile handling was to stress the organism and this stress acted to reduce the animals' responsiveness to other stressor agents later in life. Seymour Levine (26), who also studied infantile handling, found that non-handled animals were more profoundly affected by stress - both psychological such as is induced by bell ringing, light flashing, and confinement, and physiological as produced by burns, infection and muscle fatigue.

Stress and cholesterol

It has been frequently demonstrated that certain forms of stress can elevate serum cholesterol. In a study of 55 male subjects, Kuhl et al. (25) found brief immersion in cold water was followed by an immediate and significant increase in serum cholesterol. Wertlake et al. (37) studied 44 male students during periods in which they were free of examinations as well as during an examination week. The purpose of this study was to determine what possible effect mental and emotional stress might have on the level of serum cholesterol. A significant increase in mean value for serum cholesterol accompanied the stress of the examinations. These data coincide with changes which Selye (35) described in connection with the "resistance phase" of the general adaptive response to various forms of systemic stress.

Uhley and Friedman (36) conducted a study in which a group of rats fed a diet high in fat and cholesterol was exposed to a particular form of stress induced by periodic electric charging of their cages. During the first four months the average serum cholesterol and phospholipids of the rats exposed to the electrically charged cage were not significantly different from those of the control rats. The blood samples obtained from the animals at the tenth and final month of the experiment, however, were found to have considerable differences in lipid concentrations, with the average cholesterol content of blood from the experimental rats 47% greater than that of the control rats. The conclusion was that with a diet designed to produce atherosclerosis, chronic stress aggravated the process.

The high incidences of atherosclerosis among business executives and others under constant pressure has led many persons to speculate that emotional stress may be an important factor in atherogenesis. Only recently, however, has there been any acceptable evidence to support this concept. Friedman and co-workers (13) reported increases in serum cholesterol levels in men subjected to occupational stress.

Workers have consistently found periods of undue stress result in elevation of serum cholesterol levels, but the mechanism of this elevation remains unexplained.

Stress and lipoproteins

Grundy and Griffen (16) used students subjected to the

stress of final exams and found together with the increase in serum cholesterol levels, a significant (50%) increase in the S_F 12-400 class of lipoproteins.

It is generally agreed [Friedman et al. (13), Gero et al. (14), Gofman et al. (15), Jurtshuk et al. (23)] that the characteristic serum-lipid alteration in atherosclerosis is an increase (both relative and absolute) in the level of the low-density B-lipoprotein fraction. According to the "filtration" theory of atherogenesis, deposition of cholesterol in the arterial wall is enhanced by plasma-lipoprotein alterations and by changes in the subendothelial ground substances.

Gero and his co-workers (14) attempted to influence the course of atherosclerosis, induced by cholesterol feeding, by immunization with B-lipoprotein. Ten cocks and ten rabbits were given weekly intramuscular injections of an alum precipitated antigen containing 22.5 mg protein. When the immunization schedule was finished, the immunized group of rabbits and an equal number of non-immunized rabbits were fed 1 g cholesterol daily for sixteen weeks, the cocks received 2 g daily. During this time the immunized group received two more antigen injections. The immunization period itself was followed by a rise in plasma cholesterol; but the level after cholesterol feeding showed marked differences between the immunized and non-immunized groups - the former having significantly lower cholesterol levels than the latter. Immunization with B-lipoprotein was found to inhibit the

development of experimental atherosclerosis in both species of experimental animals.

B. Relation of Stress and Protein Requirements

Although concerned with purely physiological stresses (starvation and thirst), the work of Baur and Filer (2) lends some support to the hypotheses put forth in the present experiment. They found that the ability to survive stress in the form of starvation, thirst, or both, is influenced by body composition and prior plane of protein nutrition. Furthermore, under all three types of stress the rate of protein catabolism was two to three times greater for those animals fed a high protein diet than for the animals fed a low protein diet. The authors concluded the body catabolically accustomed to a higher protein intake is unable to gear down its metabolic processes when suddenly faced with a dietary emergency.

C. Effect of Protein on Serum Cholesterol Concentration

The first demonstration of an anti-atherogenic influence of dietary protein came as a result of the work of Katz, Stamler and Pick (24). Their experiments showed that high-protein supplementation suppressed hypercholesterolemia and atherogenesis in cockerels ingesting either a purified or commercial ration high in cholesterol and fat. In studies on the mechanism of this phenomenon, it was shown that vitamin supplementation enhanced the cholesterol reducing activity

of the increased protein, but vitamin supplementation alone was without influence. On the other hand, high-protein alone apparently suppressed coronary atherogenesis in a manner similar to the high-protein, high-vitamin combination. However, high-protein diet alone apparently had less definitive and clear cut effects than the combined high-protein diet with vitamin supplementation.

There have been several reports [Moyer et al. (29), Mann et al. (27), Jones and Huffman (22)] concerning effects of dietary protein on serum cholesterol concentration and recently the subject has begun to attract great attention. In 1958, Nath, Harper, and Elvehjem (30) undertook a study of the effects of various dietary levels of some proteins of both animal and vegetable origin on serum cholesterol concentration in the rat. A comparison of the effects of casein and wheat gluten indicated both the level and nature of the dietary protein influenced serum cholesterol in this species. The results showed with casein as the protein source, serum cholesterol varied inversely with increasing protein levels up to a level of 40% casein in the diet. Increasing the casein level beyond 40%, however, led to a corresponding increase in serum cholesterol levels.

When wheat gluten replaced casein as the dietary protein, the relationship between the level of protein and serum cholesterol concentration was somewhat different. In contrast to the trend with higher levels of casein, each increase in

the level of wheat gluten from 10% to 68.5% of the diet caused a progressive lowering of serum cholesterol levels. Also, serum cholesterol values for rats fed the diets containing wheat gluten were lower than values for rats fed the diets containing casein at each level of dietary protein at which the comparison was made.

The relationship of a dietary amino acid deficiency to defects of cholesterol metabolism was suggested to Fillios and Mann (27) by the preventive action of either DL-methionine or L-cystine supplementation to sulfur amino acid-deficient diets made with soybean protein and by the curative action of DL-methionine supplements given to monkeys with established hypercholesterolemia. In a study exploring the effect of diets made with soybean protein on cholesterolemia in the mouse and rat, Fillios and Mann (10) found when a protein deficient in sulfur amino acids was substituted for casein in the diet, hypercholesterolemia was demonstrated.

Further investigation by Fillios, Andrus, Mann and Stare (11) lent support to the theory of an existing relationship between dietary protein and serum cholesterol. They found the hypercholesterolemic response among rats varied according to the dietary protein level, with the lowest response observed among those animals receiving the highest level of dietary protein. However, there was no significant difference in cholesterolemic response between groups in which the protein intake did not vary greatly. Thus, the animals

receiving 10% casein in the diet did not show significantly different levels of serum cholesterol from the animals on the 20% casein diet.

Nishida, Ueno, and Kummerow (31) showed the serum cholesterol level in chicks kept on a low protein diet was significantly elevated as compared with those on a high protein diet. They proposed in a later study (32) that a low dietary protein level may impair directly or indirectly the enzymatic systems involved in cholesterol catabolism due to a combination of the following five possibilities: (1) a low protein diet may partially impair some enzyme system involved in cholesterol catabolism, (2) a low protein diet may inhibit the production of some coenzymes or cofactors necessary for cholesterol catabolism, (3) if cholesterol is assumed to be mobilized for catabolism in the form of lipoproteins, a low protein diet may produce less protein than necessary for mobilization, which may result in lowered cholesterol catabolism, (4) low protein may produce less taurine which is necessary to conjugate free bile acids, and, (5) as the metabolism of essential fatty acids seems to be correlated with cholesterol catabolism in vivo, a low-protein diet may impair the metabolism of essential fatty acids in liver, which may indirectly decrease the rate of cholesterol catabolism.

EXPERIMENTAL

A. Diets and Experimental Design

Forty male, weanling, albino rats of the Sprague-Dawley strain were divided into four groups of ten animals each for the experiment. The four groups of animals were treated as follows: (1) two groups were subjected to the flashing of 16-60 watt lights at irregular intervals for 12 hours each night; one of these groups was fed a 20% casein diet (Group D), and the other a 9% casein diet (Group B); (2) the other two groups were likewise differentiated by the level of protein intake - Group A, 9% casein and Group C, 20% casein. The experimental groups (exposed to flashing light) were in one section of the room with their cages enclosed by a wooden frame to which a heavy canvas covering was nailed. The canvas was rolled up when the lights were not in operation, and let down when the lights were flashing. The control animals, in another section of the room, were in a similar canvas enclosure so that both experimental and control animals were subjected to a 2 degree rise in temperature during the night. The control animals were thus shielded from the lights by two thicknesses of canvas and the distance between the two enclosures.

The diets provided the following nutrients expressed per 100 gm diet:

Low Protein Diet[1]		High Protein Diet[1]	
Casein	9.0	Casein	20.0
Salt Mixture W[2]	4.0	Salt Mixture W[2]	4.0
Choline	0.15	Choline	0.15
Corn Oil	5.0	Corn Oil	5.0
Sucrose	80.84	Sucrose	70.60
DL-Methionine	0.30	Vitamin Mix	0.25
DL-Tryptophan	0.10		
DL-Threonine	0.36		
Vitamin Mix	0.25		

The vitamin mix[1] contained the following in milligrams per 100 gm diet:

Vitamin A Powder (20,000 IU/gm)	10.0
Niacin	1.0
Calciferol	0.18
Thiamine HCl	0.5
Riboflavin	0.5
Pyridoxine	0.25
Calcium Pantothenate	2.0
Inositol	10.0
Folic Acid	0.02
Vitamin B_{12} (0.1% trituration with mannitol)	2.0
Biotin	0.01
Para-aminobenzoic Acid	1.0
Menadione	0.38
Sucrose	222.16

The initial average weight of any one group of rats did not vary from that of any other group of rats by more than one gram. The animals were housed individually in metabolism cages. Food and water were provided ad libitum to all animals and records were kept of each animal's food intake and weight gain. Urine and feces were collected during the last

1. Harper, A.E. 1959 Sequence in which amino acids of casein are limiting for rat growth. J. Nutrition, 67:109.
2. Wesson modification of Osborne and Mendel salt mixture. Science, 75:339, 1932. Obtained from Nutritional Biochemicals Corp.

two weeks of the experiment to be used in the calculation of nitrogen balance. On the 28th day, the animals were anesthetized and blood samples obtained from tail snips. Thereafter, they were decapitated and their livers and adrenal glands removed for purposes of the chemical analyses to be described below.

B. Chemical Analyses

Serum Cholesterol Determinations: Total and free cholesterol in the blood sera were determined by a modified Schoenheimer-Sperry method[1]. Serum samples of 0.2 ml were pipetted into a 5 ml volumetric flask containing about 3 ml of 1:1 acetone-alcohol. The mixture was heated, agitated, cooled and made to volume with acetone-alcohol. The protein, denatured by the solvent and heat treatment, was removed by filtration through fluted, fat-free filter paper. One ml of the filtrate was pipetted directly into 5 ml centrifuge tubes for the total cholesterol; 2 ml of this filtrate were pipetted directly into 5 ml centrifuge tubes for the free cholesterol, and 50% KOH was added to each tube for saponification and consequent release of cholesterol from its esters. For both total and free cholesterol, 2 ml of a solution of 0.5% alcoholic digitonin were added to the acidified extracts (alcoholic acetic acid). The precipitated digitonide was allowed to flocculate in a water bath at 60° C for about 30 minutes, and was then stored in the dark at room

1. Schoenheimer, R. and W.M. Sperry 1934 A micro determination of free and combined cholesterol. J. Biol. Chem., 106:745.

temperature for 48 hours. The tubes containing the cholesterol digitonide were centrifuged for 30 minutes at about 1500 r.p.m. and the supernatant was decanted. The precipitate was washed again with 1:1 acetone-ether, recentrifuged for 15 minutes, and the supernatant again decanted. The washing process was repeated two more times with anhydrous ether. The centrifuge tubes were placed in a sand bath in an oven at 110-115° C for 30 minutes and each precipitate was then dissolved in 1 ml of glacial acetic acid while still hot. After cooling, 2 ml of cold Liebermann-Burchard reagent (1:20 sulfuric acid and acetic anhydride) were added to each tube. After allowing 27 minutes for the development of color, the optical densities of the cholesterol solutions were read in a Beckman DB Spectrophotometer at 620 mu. Cholesterol concentrations were determined by comparison with a standard curve.

The mg of total serum cholesterol per 100 cc of blood was obtained by multiplying the reading on the standard curve by a factor of 2500, and a factor of 1250 was used for determining mg of free serum cholesterol per 100 cc of blood. The factors were obtained thus:

$$\text{factor for total} = \frac{5 \text{ ml (total vol. of extract)}}{0.2 \text{ ml (vol. serum)}} \times \frac{100}{1 \text{ ml (sample)}} = 2500$$

$$\text{factor for free} = \frac{5 \text{ ml (total vol. of extract}}{0.2 \text{ ml (vol. serum)}} \times \frac{100}{2 \text{ ml (sample)}} = 1250$$

sample calculation:
A_1 - Beckman reading: total, .044; free, .021
standard curve reading for total cholesterol: .029
standard curve reading for free cholesterol: .014
total mg cholesterol % = .029 x 2500 = 72.5
free mg cholesterol % = .014 x 1250 = 17.5

Liver Glutathione: Method of Woodward and Fry (39). The livers, which had been stored frozen, were thawed at room temperature for about 2 hours. A 5 gm portion was homogenized with 5 ml of 4% sulfosalicylic acid in a Waring homogenizer. The contents were decanted into a 50 ml volumetric flask and the residue was again homogenized with 10 ml of 2% sulfosalicylic acid. The contents were quantitatively transferred to the 50 ml volumetric flask containing the original extract. The volumetric flask was then filled to volume with 2% sulfosalicylic acid. The mixture was shaken and allowed to stand for 30 minutes whereupon an extract was obtained by centrifuging and decanting the supernatant. A 10 ml aliquot of the sulfosalicylic acid extract was placed in a 50 ml Erlenmeyer flask. To this sample were added 2.5 ml of 4% sulfosalicylic acid, 2.5 ml of 5% potassium iodide, and 2 drops of starch solution. A blank was prepared in a second 50 ml Erlenmeyer flask by adding the same reagents to 10 ml of 2% sulfosalicylic acid. The flask containing the sample was placed against a white background in a water bath containing ice and water. A microburet was used to add 0.001 N potassium iodate to the sample and the end point identified when there was a persistent blue color. The blank was treated in a similar manner. In order to determine the ascorbic acid

content of the samples, a second titration series was carried out. A 10 ml aliquot of the sulfosalicylic acid extract was placed in a 50 ml Erlenmeyer flask, while a blank consisting of 10 ml of 2% sulfosalicylic acid was prepared in a second flask. The sample, blank, and 10 ml of standard ascorbic acid solution were titrated with 2,6-dichlorophenolindophenol reagent using a microburet. The end point was reached when there was a faint pink color that persisted for 2 minutes or more.

The uncorrected glutathione concentration for 10 ml of sulfosalicylic acid filtrate (in milligrams per 100 gm) was obtained by dividing 100 times the volume (ml) of 0.001 N potassium iodate (sample minue blank) used by 3.26 which is the theoretical titer for 1 mg of glutathione. Then 3.5 times the ascorbic acid concentration (100 times the volume in ml of 2,6-dichlorophenolindophenol - sample minus blank- used divided by the titration volume for the same amount of standard ascorbic acid solution) was subtracted from the uncorrected glutathione value in order to obtain the corrected glutathione concentration.

sample calculation:

A_1 - weight of liver sample: 5.00 gm
titer of KIO_4: 4.14 ml
titer of 2,6-dichlorophenolindophenol: 1.73 ml
blank titer of KIO_4: .05 ml
blank titer of 2,6-dichlorophenolindophenol: .04 ml
titration of standard ascorbic acid with 2,6-dichlorophenolindophenol: 10.50 ml

$$\frac{100 \times (4.14-.05)}{3.26} - 3.5 \times \frac{(100 \times (1.73-.04))}{10.50} = 69.11 \text{ mg glutathione per 100 ml extract}$$

Nitrogen Determination: Urinary and fecal nitrogen were determined by the macro Kjeldahl technique.

C. Statistical Analysis

Standard error of the means were calculated on all data and Student's "t" test was used as a measure of significance.

RESULTS

A. Effects of Stress with Varying Levels of Protein

The flashing of 16-60 watt bulbs into the cages of male, albino rats from a distance of two and one half feet at irregular intervals, while not affecting food intake significantly as is seen by comparison with the food intake of the control animals (Groups A and C), had a marked effect on the growth of the low-protein, stressed animals (Group B). The growth of the high-protein stressed animals (Group D) was not affected (table 1, figure 1).

Table 1. Influence of flashing light stress on growth and food intake in albino rats receiving high or low protein diets.

Group	Stress	Diet	Food Intake[1] gm/week	Wt. Gain[2] gm/week	Wt. Gain gm/4 weeks
A	No	9% Casein	81±2*	30±1	123±4
B	Yes	9% Casein	75±2	25±1	98±3
C	No	20% Casein	90±0	43±0	170±2
D	Yes	20% Casein	90±0	43±0	173±2

* Standard error of the mean

[1] Level of statistical significance: A vs B, none; C vs D, none; A vs C, 1%; B vs D, 1%.

[2] Level of statistical significance: A vs B, 1%; C vs D, none; A vs C, 1%; B vs D, 1%.

All groups were found to be in positive nitrogen balance

(table 2, figure 2); liver glutathione was found to increase with protein level and stress (table 3, figure 3). Total serum cholesterol levels did not differ significantly among any of the groups (table 3, figure 4) but free serum cholesterol was found to be highest in the low-protein, stressed animals, and lowest in the high-protein, stressed animals (table 3, figure 5).

Table 2. Nitrogen retention in the albino rat as affected by the stress of flashing lights with high and low protein diets.

Group	Stress	Diet	Mg Nitrogen[1] retained/day
A	No	9% Casein	169±4*
B	Yes	9% Casein	158±5
C	No	20% Casein	260±6
D	Yes	20% Casein	306±6

* Standard error of the mean
1 Level of statistical significance: A vs B, none; C vs D, 1%; A vs C, 1%; B vs D, 1%

Table 3. Influence of flashing light stress on cholesterol and glutathione levels in albino rats on high and low protein diets.

Group	Stress	Diet	Liver Glutathione[1] mg/100 gm	Total Serum Cholesterol mg/100 cc[2]	Free Serum Cholesterol mg/100 cc[3]
A	No	9% Casein	55.7±4.0*	83.3±5.3	22.2±1.1
B	Yes	9% Casein	104.1±2.0	94.3±4.0	30.0±1.7
C	No	20% Casein	134.2±6.7	94.8±5.4	21.0±0.5
D	Yes	20% Casein	160.9±5.3	81.8±4.0	14.2±0.2

* Standard error of the mean
1 Level of statistical significance: A vs B, 1%; C vs D, 1%; A vs C, 1%; B vs D, 1%
2 Level of statistical significance: A vs B, none; C vs D, none; A vs C, none; B vs D, 5%
3 Level of statistical significance: A vs B, 1%; C vs D, 1%; A vs C, none; B vs D, 1%

The low-protein, stressed animals had the largest adrenal glands (weight per 100 g of body weight); the non-stressed, low-protein group had the next largest, and the high-protein groups had the smallest (table 4, figure 6). The low-protein, stressed animals had the smallest livers per 100 g of body weight; the livers of the other groups did not differ significantly (table 4, figure 7).

Table 4. Adrenal gland weights in albino rats subject and not subject to the stress of flashing lights while fed high or low protein diets.

Group	Stress	Diet	Adrenal Gland Wt.[1] gm/100 gm of body weight	Liver Wt.[2] gm/100 gm of body weight
A	No	9% Casein	18.1±0.8*	5.0±0.1
B	Yes	9% Casein	22.4±0.9	4.1±0.1
C	No	20% Casein	14.9±1.0	5.2±0.1
D	Yes	20% Casein	15.4±0.6	5.0±0.1

* Standard error of the mean

[1] Level of statistical significance: A vs B, 1%; C vs D, none; A vs C, none; B vs D, 1%

[2] Level of statistical significance: A vs B, 1%; C vs D, none; A vs C, none; B vs D, 1%

B. Effect of Varying Protein Levels

When the high and low protein control groups were compared with each other (Groups C and A), the expected results were noted. Rats fed the 20% protein diet consumed significantly greater amounts of food and gained a significantly greater amount of weight (table 1). The rats fed the 20%

protein diet retained more nitrogen (table 2) and had higher levels of liver glutathione than did those animals fed 9% casein (table 2). Total and free serum cholesterol (table 3) and adrenal gland weights (table 4) did not vary significantly between the two groups. Liver weights per 100 gm body weight also did not vary between the two groups (table 4).

When the high and low protein stressed groups were compared with each other (Groups D and B), weight differences were again noted; the high protein group weighed considerably more than the low protein group (table 1). The stressed rats fed the high protein diet retained more nitrogen (table 2) - almost twice as much - and had higher levels of liver glutathione (table 3) than did the animals receiving the low protein diet. Unlike the comparison of the controls, however, the comparison of the stressed groups revealed the low-protein stressed rats (Group B) had significantly higher total and free serum cholesterol levels than the high-protein stressed rats (Group D, table 3). Group B also had larger adrenal glands and smaller livers than group D (table 4).

C. - Graphical representation of the results

Figure 1. Influence of stress on growth of male, albino rats fed verying levels of protein (casein) in the diet. (Weanling rats sacrificed after 28 days on experiment.)

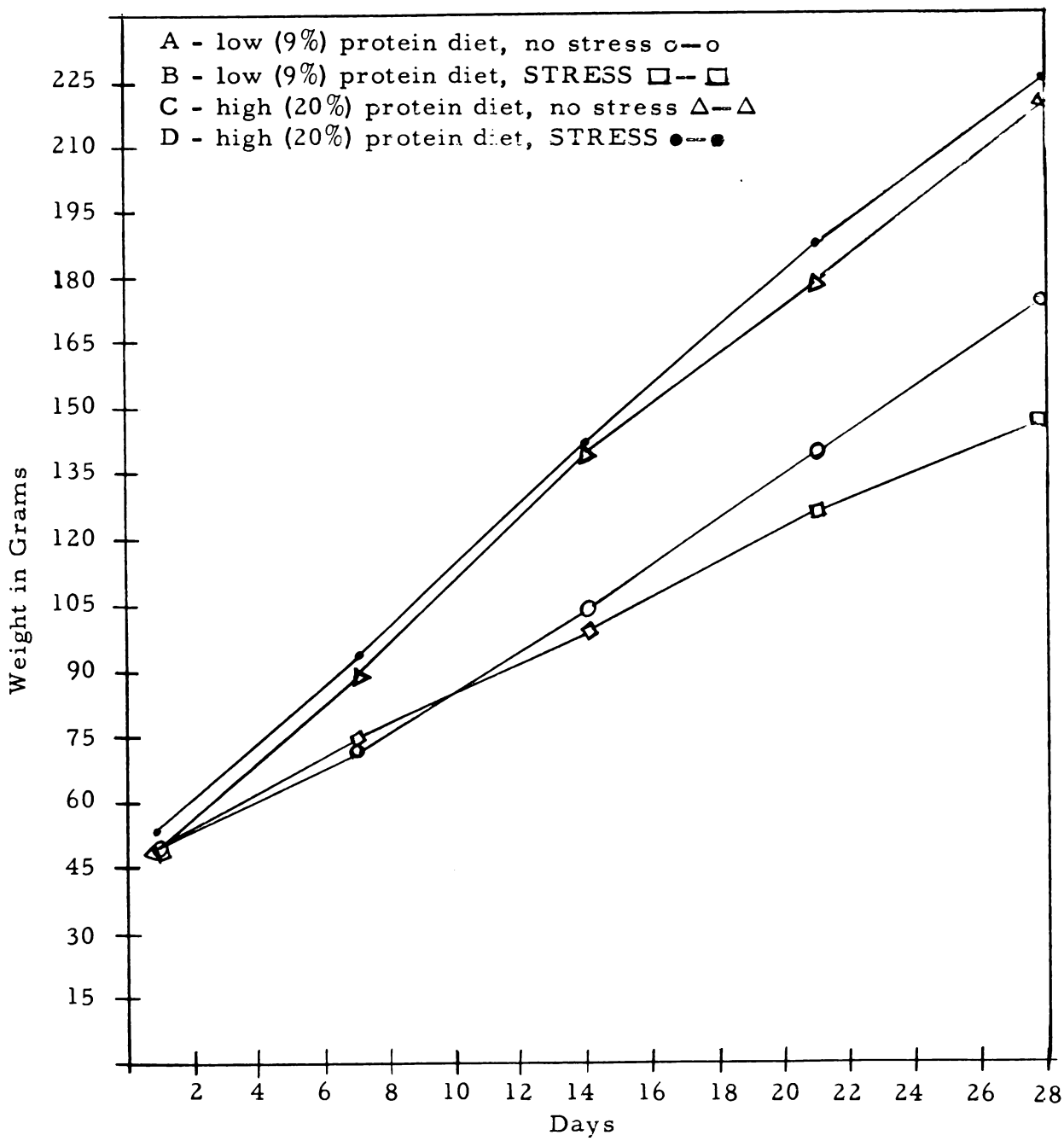


Figure 2. Influence of stress on nitrogen retention of male, albino rats fed varying levels of protein (casein) in the diet. (Weanling rats sacrificed after 28 days on the experiment.)

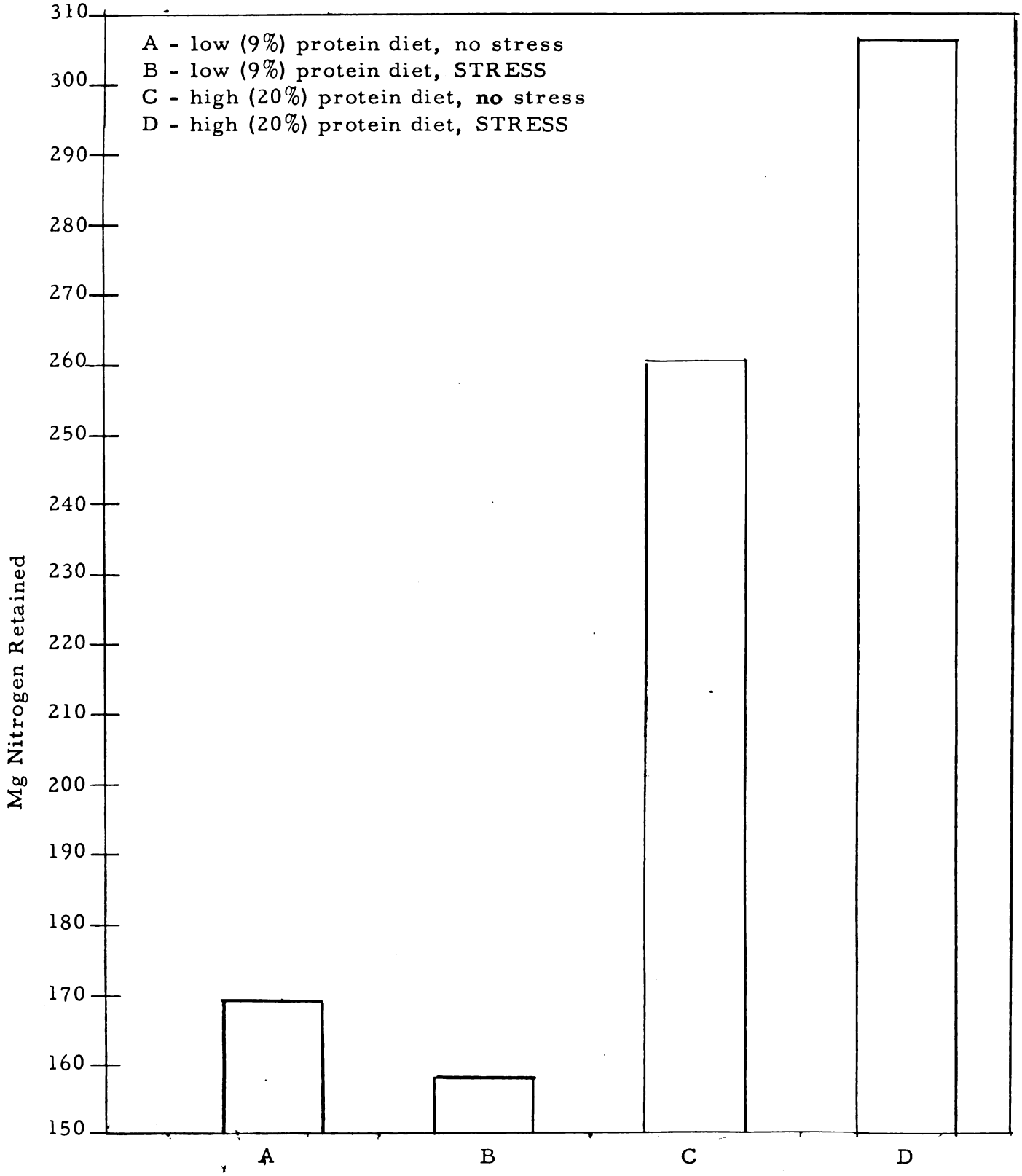


Figure 3. Influence of stress on liver glutathione levels of male, albino rats fed varying levels of protein (casein) in the diet. (Weanling rats sacrificed after 28 days on experiment.)

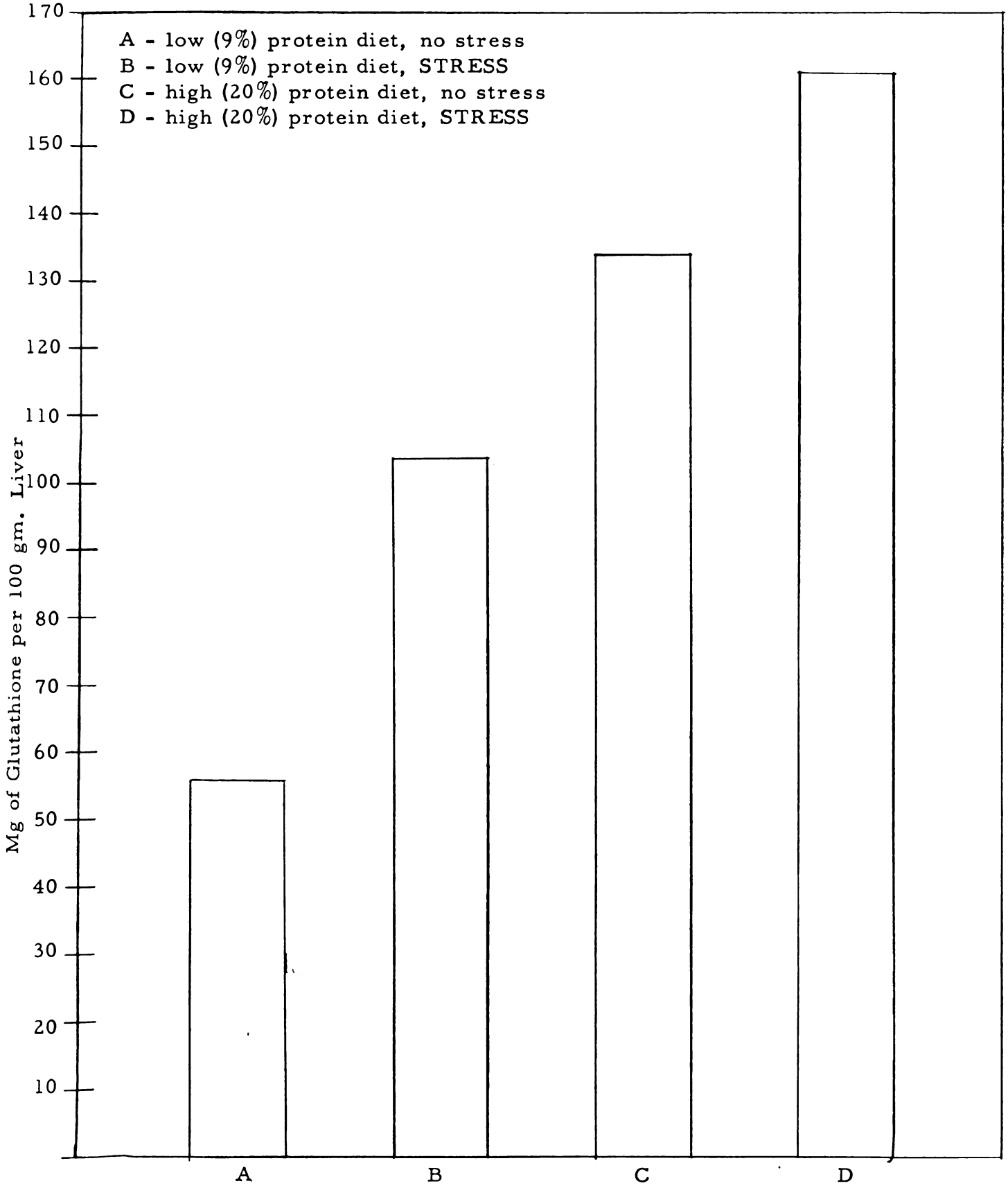


Figure 4. Influence of stress on total serum cholesterol levels of male, albino rats fed varying levels of protein (casein) in the diet. (Weanling rats sacrificed after 28 days on the experiment.)

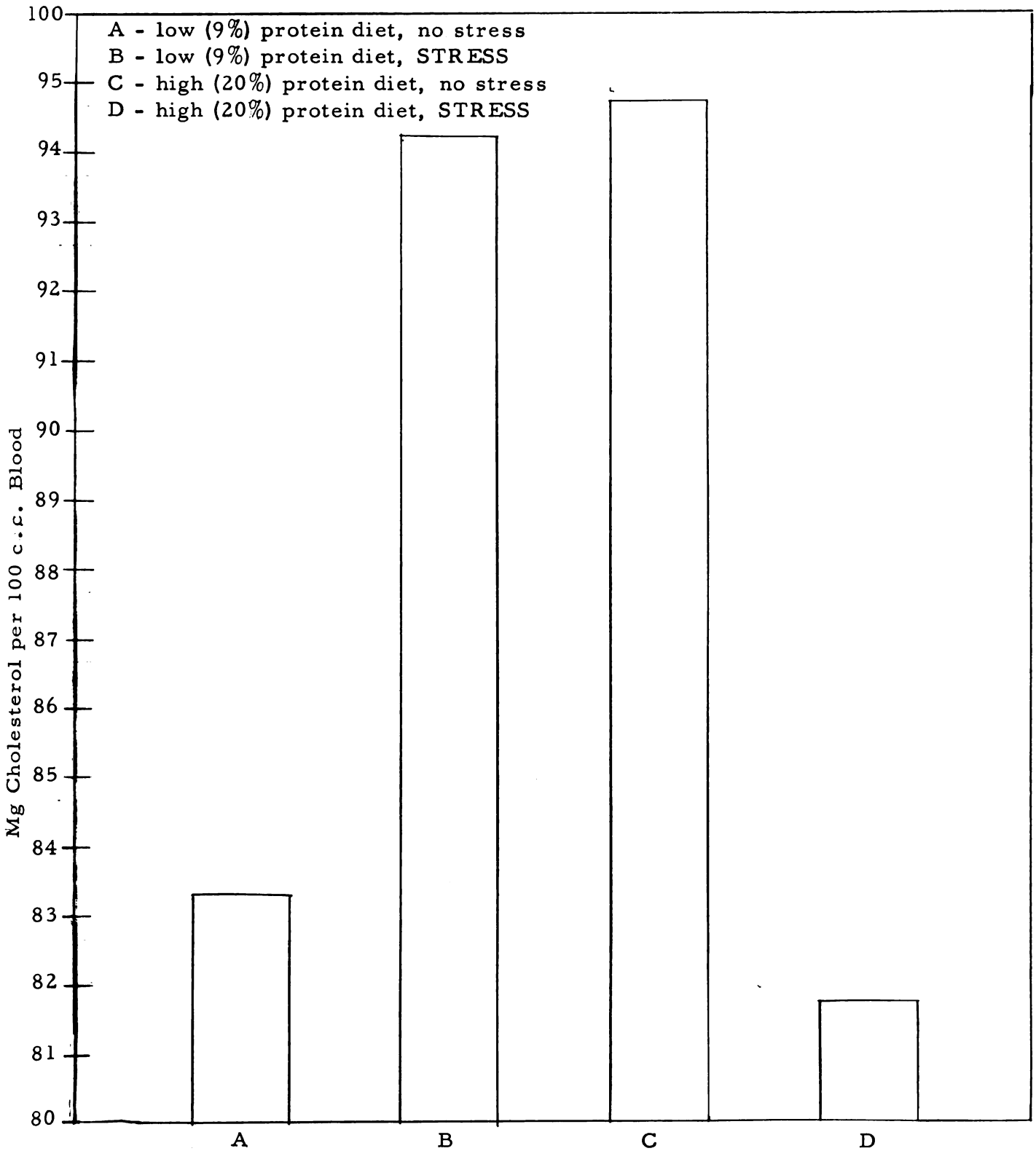


Figure 5. Influence of stress on free serum cholesterol levels of male, albino rats fed varying levels of protein (casein) in the diet. (Weanling rats sacrificed after 28 days on the experiment.)

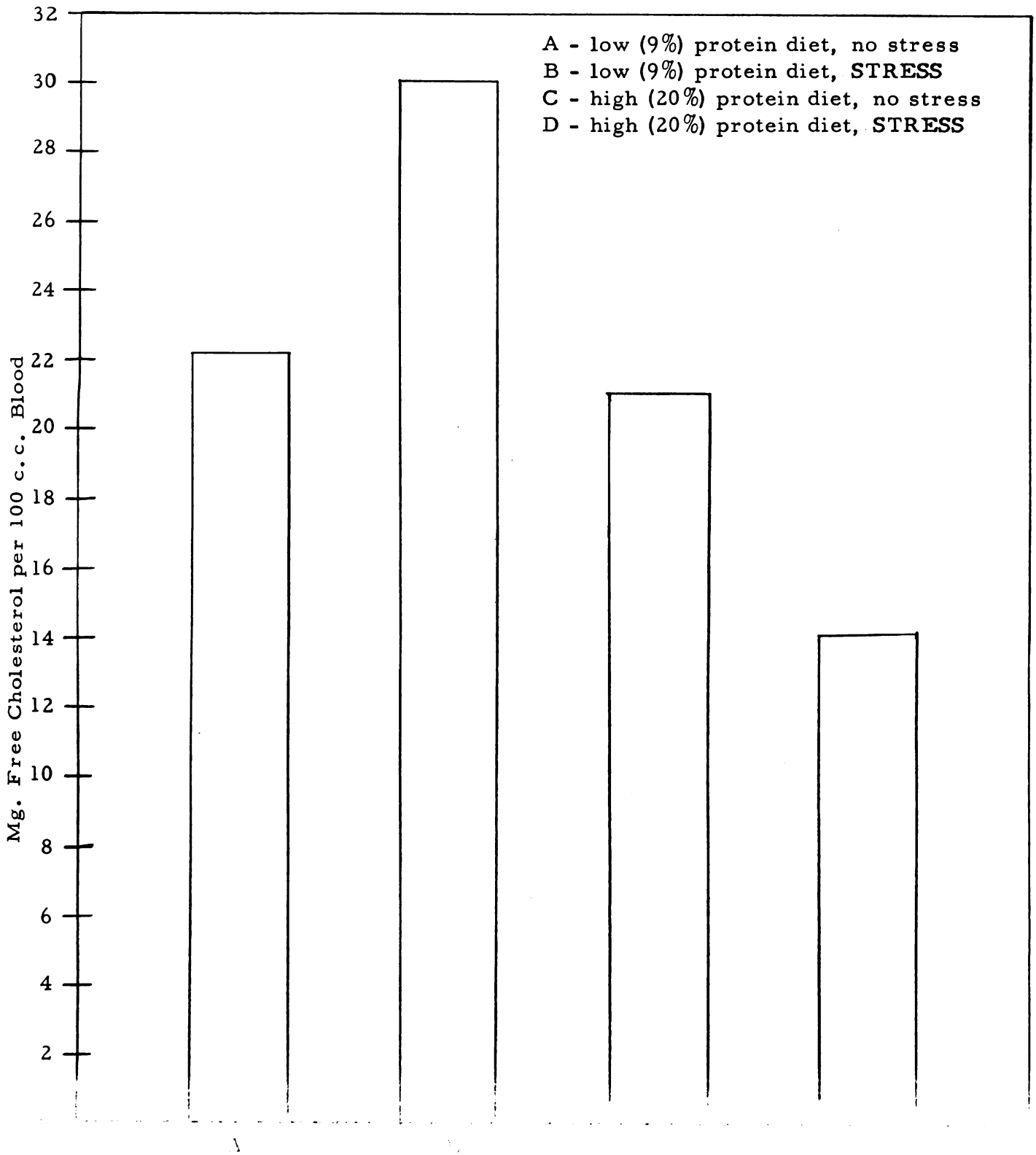


Figure 6. Influence of stress on adrenal gland weights of male, albino rats fed varying levels of protein (casein) in the diet. (Weanling rats sacrificed after 28 days on the experiment.)

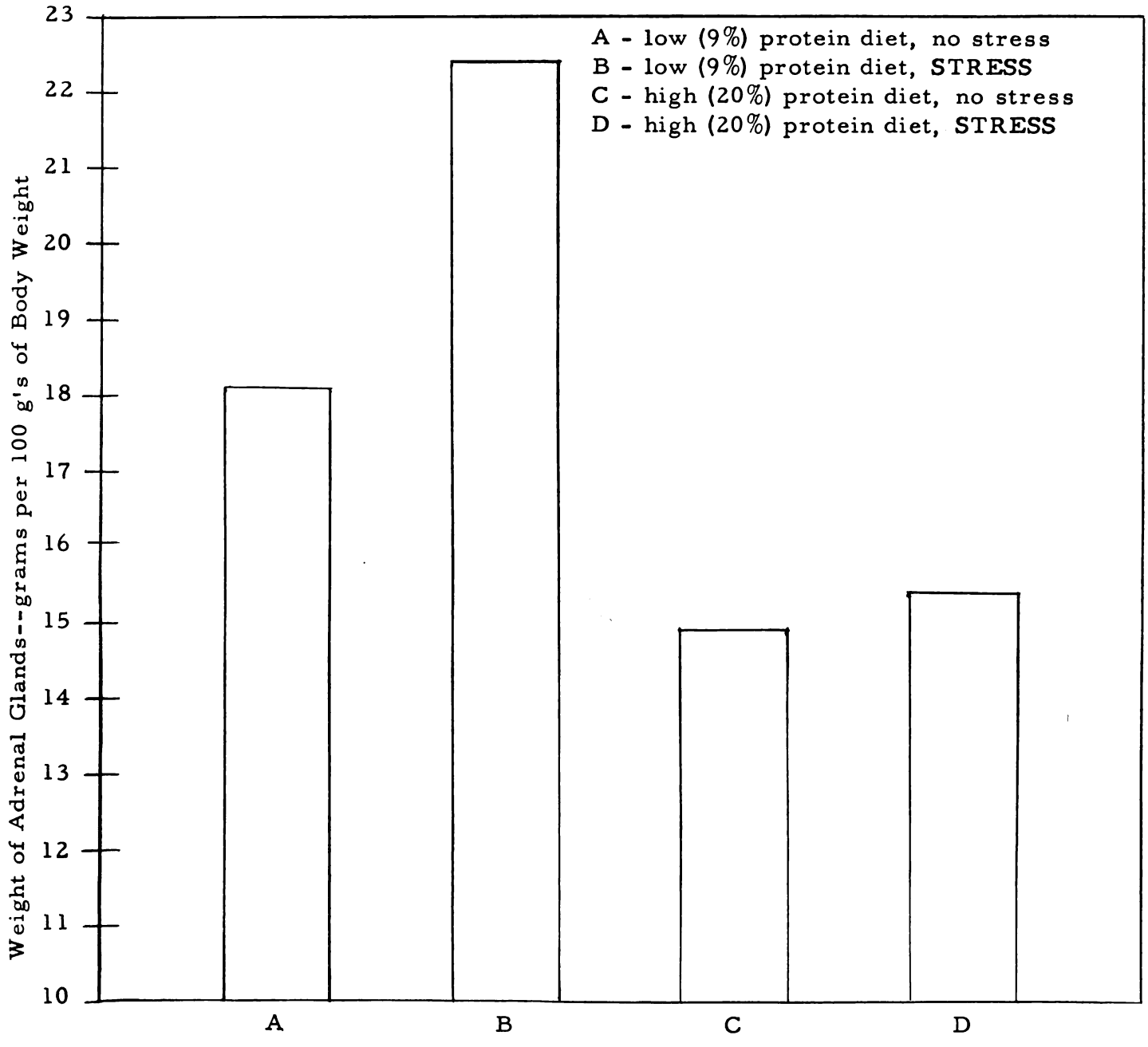
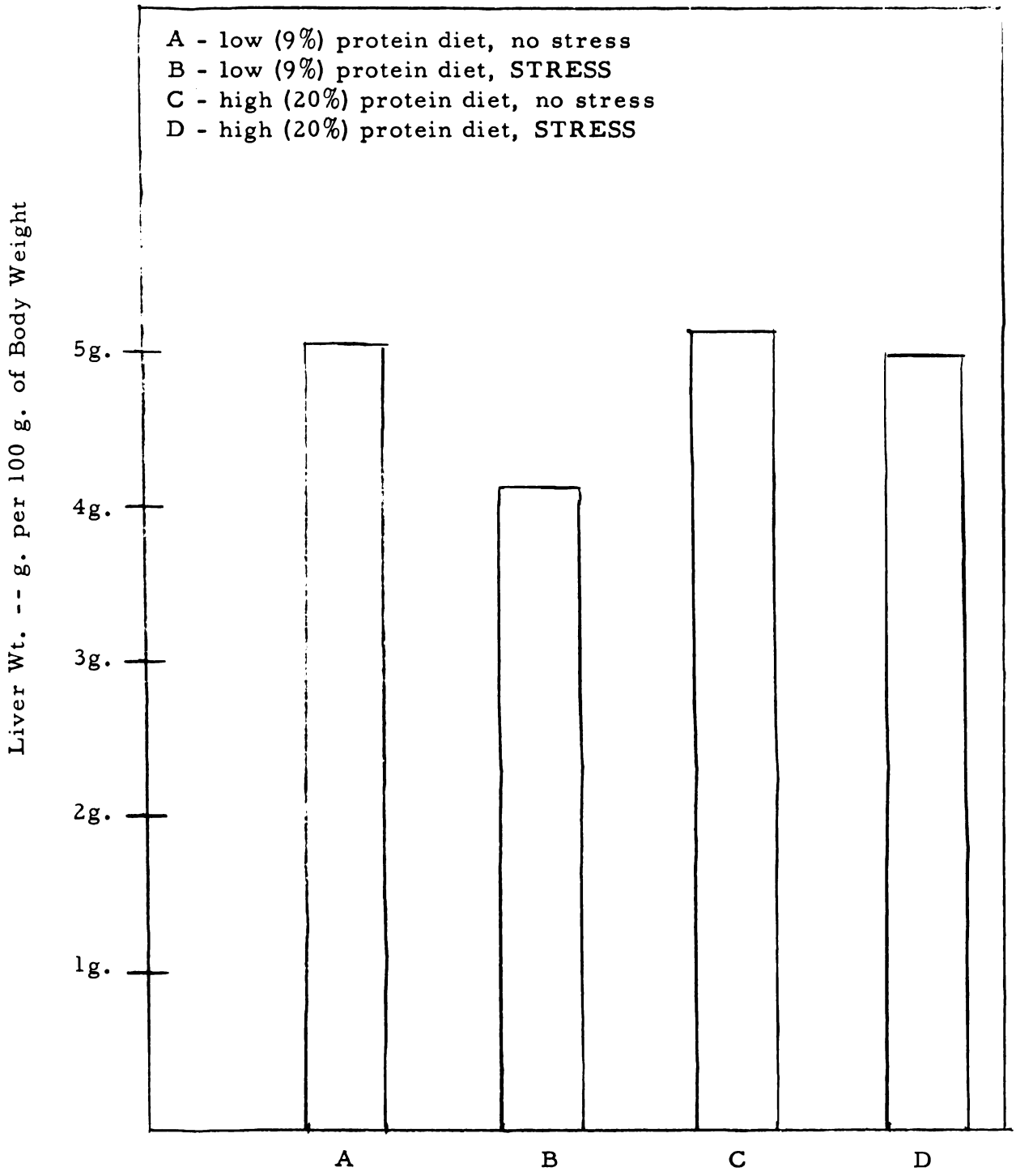


Figure 7. Influence of flashing lights on liver weights of male, albino rats fed varying levels of protein (casein) in the diet. (Weanling rats sacrificed after 28 days on experiment.)



DISCUSSION

A. Effects of Stress with Varying Levels of Protein

1. Influence of stress on growth

Introducing the stress of flashing lights to rats receiving a diet containing 9% casein as the protein source markedly reduced the growth of male albino rats as is evidenced by comparison of the growth of these animals with the growth of rats receiving the same diet but not subjected to the stress. This growth reduction occurred despite the fact that there was no significant difference in food intake between the two groups. It should be noted, however, that the difference in food intake and difference in growth between the two groups are of the same magnitude. The fact that the food intake differences are not statistically significant is undoubtedly influenced by the difficulties in obtaining exact measurements of food intake. It should be considered, therefore, that the reduced food intake on the part of the stressed, low-protein animals had some bearing on the reduced growth rate. The low level of protein also influenced the restriction of growth as is evidenced by the fact that the low-protein, non-stressed group gained significantly less weight than did the high-protein, non-stressed group. However, in this latter case there is also a difference in food consumption to be considered, for the low protein group ate considerably less food than did the high protein group.

An examination of data in the two high-protein groups reveals that the introduction of the lights had no influence on the growth rate of the rats - both stressed and non-stressed groups attaining the same level of growth.

In view of the reports by Chaloupka et al. (6) indicating preferential use of limiting amounts of amino acids, it seems in this study the preferential use of amino acids might be an important factor in the reduced growth rate of the stressed animals receiving the low protein diet. Chaloupka reported the first requirement met by amino acids was for the maintenance of nitrogen balance and growth requirements were fulfilled after this. Assuming protein catabolism to be increased by stress, then Group B (low-protein, stress) would have a greater requirement for amino acids to maintain positive nitrogen balance (since these were growing animals) than would Group A (low-protein, non-stress). Hence the supply of amino acids for growth in Group B would be decreased, resulting in the observed decreased growth rates in these animals. Also, the increased levels of the nitrogen-containing compound, glutathione, in the liver indicate further increase in nitrogen requirements which would limit the supply of nitrogen available for growth.

Ample supplies of amino acids were available to animals on the high protein diets to satisfy the requirements for both maintenance of positive nitrogen balance and growth.

2. Influence of stress on nitrogen retention

While all groups remained in positive nitrogen balance, as is mandatory with the growing animal, it can be seen that the low-protein, stressed animals showed the lowest degree of positive balance, and the high-protein, stressed animals the highest. Chaloupka's theory on preferential distribution of amino acids offers a possible explanation for the variation in nitrogen retention among the four groups. The non-stressed group (A) apparently had sufficient protein to keep them in positive nitrogen balance and to promote growth at a moderate, if somewhat limited, rate. The stressed group (B) on this diet, while able to maintain positive nitrogen balance, had markedly reduced growth rates.

Group C - the animals receiving the 20% casein diet and not subjected to the light - apparently was receiving more than adequate supplies of essential amino acids. These animals were kept in a state of positive nitrogen balance, grew at a healthy rate, and excreted the excess nitrogen. Group D, however, receiving the same diet but subjected to the lights, while maintaining positive nitrogen balance and growing just as well as its non-stressed counterpart, retained more nitrogen than did Group C and it is suggested that this difference in nitrogen retention reflects an increased need for amino acids established by the stress of the flashing lights. The mechanism whereby the stress caused an increased amino acid requirement was not elucidated in this particular study.

3. Influence of stress on liver glutathione levels

Although no confirmation of this theory can be found in the literature, it would not seem unreasonable to suggest liver glutathione levels increase with an increase in dietary protein. Jurtshuk et al. (23) found blood glutathione levels dropped significantly when the animal was exposed to intense auditory stimulation - but whether an inverse relationship exists between blood glutathione levels and liver glutathione levels has not been established. The stressed animals in this experiment showed significantly increased levels of liver glutathione. It is a possibility that stress inhibits mobilization of glutathione out of the liver.

4. Influence of stress on adrenal gland weights

The animals with the heaviest adrenal glands per 100 gm of body weight were those stressed animals receiving the low protein diet. Evidence that the low protein nature of the diet also contributed to the general stress of the animals is offered by the fact that the adrenal glands of the non-stressed animals on the low protein diet were considerably larger than those of the non-stressed animals on the high-protein diet. Moreover, the differences in adrenal size between the two non-stressed groups were not significant while the differences between the two stressed groups were significant at the 1% level, indicating the combination of low protein plus the stress of the lights had a greater effect on adrenal gland size than did the stress of the low

protein diet alone.

5. Influence of stress on serum cholesterol levels

The total serum cholesterol levels of the rats subjected to the stress of the flashing lights and fed a diet containing 9% casein were not significantly higher than those of animals fed the same diet but not exposed to the lights. There was likewise no significant difference between total serum cholesterol levels of the two high protein groups - stressed and non-stressed. These findings are in keeping with those of Uhley and Friedman (36) who found that at four months, rats subjected to the stress of electric shock had total serum cholesterol levels comparable to those of animals not receiving the shock, but at ten months the stressed rats had significantly higher levels of serum cholesterol. Again, in keeping with the findings of Fillios, Andrews, Mann and Stare (11) who found raising the level of dietary protein was effective in lowering serum cholesterol only after fifty-six days on the diet and there had to be a great difference in dietary protein levels to show a significant difference in cholesterolemic response, the two non-stressed groups - one fed 9% casein, the other fed 20% casein, did not show significant differences in total serum cholesterol levels. The two stressed groups, however, one on a high protein diet, the other on a low protein diet, showed a difference in cholesterolemic response significant at the 5% level, indicating the stress of the blinking lights coupled with the

low protein nature of the diet tended to bring about an increase in the level of total serum cholesterol that was not evident when the only difference between groups was an 11% difference in the level of dietary protein.

While the differences are not statistically significant, there is a trend for the high-protein, non-stressed group to have higher total serum cholesterol levels than the high-protein, stressed group. This might possibly be explained on the basis of efficiency of cholesterol mobilization and catabolism: the animals in Group D (high protein) as a result of the stress to which they were subjected, were operating at a greater level of efficiency, as evidenced by the fact of greater nitrogen retention and lower serum cholesterol levels.

Some interesting results were obtained with respect to the levels of free serum cholesterol: the low-protein, stressed animals had free serum cholesterol levels significantly higher than those of any of the other groups, while the high-protein, stressed animals had free serum cholesterol levels significantly lower than those of any of the other groups. In reviewing the reports of other workers, frequent suggestions are made of an association between B-lipoprotein levels and cholesterol levels. Gero et al. (14) found that rats injected with B-lipoproteins showed significantly lower cholesterol levels than those not receiving the injection, and Nishida et al. (31) stated that if cholesterol is assumed

to be mobilized for catabolism in the form of lipoproteins, a low protein diet may produce less protein than required for mobilization which could result in lowered cholesterol catabolism. In the light of these findings certain suggestions in regard to the results obtained in the present study seem appropriate.

In the first place, it is possible that the reason for the higher level of free serum cholesterol in the stressed, low-protein group is that there was insufficient protein available for cholesterol binding. The possibility exists that the resulting inefficiency in mobilization of the cholesterol for catabolism might eventually result in the deposition of the free cholesterol on the arterial walls and this possibility is in need of further investigation.

A second possibility is that Group D, stressed and receiving a diet high in protein, produced more cholesterol as a result of the stress and by retaining more nitrogen was able to facilitate mobilization and eventual catabolism of the cholesterol. In other words, Group D produced more cholesterol, as did Group B, however, unlike Group B, had sufficient protein (as a result of increased nitrogen retention) to mobilize the cholesterol in the form of lipoproteins for catabolism, resulting in a final total serum cholesterol level comparable to that of Group C (high-protein, non-stressed) and a free serum cholesterol level lower than that of Group C because of increased effeciency in cholesterol

binding.

6. Influence of stress on liver weights

Of the two stressed groups, only the one fed a low protein diet exhibited significantly smaller livers on the basis of weight per 100 gm of body weight than did its dietary counterpart. The fact that this stressed group had smaller livers than the non-stressed group could perhaps be explained on the basis of a reduction of the protein availability by the introduction of the stress. That is, the stress possibly introduced increased requirements for amino acids, thereby reducing the supply available for the synthesis of liver tissue.

B. Effect of Varying Protein Levels

When the high and low protein groups are compared with each other (Groups C and A) the results are as expected (tables 1-4). However, none of the differences are as great as those seen when the stressed groups are compared (tables 1-4). This indicates differences resulting from a variation in protein intake are augmented and compounded by the introduction of stress. In the non-stressed animals there is no significant difference in the total cholesterol levels of the low and high protein groups; in the stressed animals this difference is significant.

SUGGESTIONS FOR CONTINUATION OF THIS STUDY

Several questions have not been fully answered by the present study and several points have been raised. After verifying the results obtained in this experiment, an attempt should be made to clarify these points.

It would be of some value to use brighter lights and to try increased exposure to the lights. Other levels of casein in the diet should be used as well as other sources of protein, also at various levels.

The relationship between stress, protein, and cholesterol need further elucidation. Continuation of the experiment over a longer period of time might result in more significant reductions in blood cholesterol levels. Examination of the arterial walls for evidences of cholesterol deposition might reveal the extent of the influence of the high-protein diet.

It is suggested that adult animals be used in the study, and blood samples for cholesterol determination be taken at the beginning of the experiment as well as at the end. The reason for the increased nitrogen retention on the part of the stressed, high-protein animals should be explored. Also there should be a determination of B-lipoprotein (S_F 12-400 type) level in both the stressed and non-stressed animals at the beginning and end of the experiment. At some date the mechanism through which stress affects blood cholesterol levels should be explored.

SUMMARY AND CONCLUSIONS

The object of this study was to determine the influence of varying levels of protein on the effect of the stress of bright flashing lights on male, albino rats. Forty rats were divided into four groups of ten animals each. The four groups were housed in a non-temperature controlled, non-air-conditioned room where two of the groups were subjected to the irregular flashing of 16-60 watt bulbs at a distance of two and one half feet for twelve hours each day. Ten of these "stressed" rats were fed a diet containing 20% casein and ten received a diet containing 9% casein. The other two groups in the same room were likewise divided according to diet, but were not subjected to the stress of the flashing lights. The animals were sacrificed after 28 days and liver and blood samples were taken for analysis.

Both of the stressed groups showed larger adrenal glands than their dietary counterparts and also had higher levels of liver glutathione. The low-protein, stressed animals gained less weight and had higher free serum cholesterol levels than did their controls, while the high-protein, stressed group had lower free serum cholesterol levels and higher nitrogen retention than did its control group.

It has been postulated that the results obtained regarding growth and nitrogen retention lend support to the hypothesis that stress alters protein requirements: in the

low-protein, stressed group there was only sufficient protein to maintain positive nitrogen balance - not to support the rapid growth that was evident in the animals receiving the same diet but not subjected to the flashing lights; perhaps the stress was causing a need for extra protein to meet a specific but as yet unknown requirement. The high-protein, stressed group, however, had enough protein to promote growth, maintain nitrogen balance and meet the unknown requirements.

Lack of protein for cholesterol binding is believed to be the reason for the high level of free serum cholesterol in the stressed, low-protein animals. At the same time, the high degree of nitrogen retention in the stressed, high-protein animals is believed to account for the cholesterol situation in this group - the stress caused them to produce more cholesterol, additional nitrogen was retained to facilitate cholesterol binding and mobilization which resulted in less free serum cholesterol.

It is concluded the stress induced in rats by bright, flashing lights has a definite effect on the protein requirements of the male albino rat.

REFERENCES

1. Adams, R.L., F.N. Andrews, J.C. Rogler, and C.W. Carrick 1962 The protein requirement of 4-week old chicks as affected by temperature. J. Nutrition, 77:121.

2. Baur, L.S. and L.J. Filer, Jr. 1959 Influence of body composition of weanling pigs on survival under stress. J. Nutrition, 69:128.

3. Bernard, C. 1949 An Introduction to the Study of Experimental Medicine. Translated by Henry Copley Green. Dover Publications, Inc., New York, New York.

4. Brent, L., J. Brown, and P.B. Medawar 1958 Skin transplantation immunity in relation to hypersensitivity. Lancet, July-Dec.:561.

5. Cannon, W.B. 1922 New evidence for sympathetic control of some internal secretions. Am. J. Psychiat., 2:15.

6. Chaloupka, M.M., J.N. Williams, Jr., M.S. Reynolds, and C.A. Elvehjem 1957 Relative roles of niacin and tryptophan in maintaining blood pyridine nucleotides, nitrogen balance, and growth in adult rats. J. Nutrition, 63:361.

7. Denenberg, V.H. and G.G. Karas 1959 Effects of differential infantile handling upon weight gain and mortality in the rat and mouse. Science, 130:629.

8. Ershoff, B.H. 1955 Nutrition and stress. Nutrition Reviews, 13:33.

9. Filer, L.J., Jr. 1961 IN E. Halac, Jr.: Effects of stress on animals fed high protein diets. Am. J. Clinical Nutrition, 9:560.

10. Fillios, L.C. and G.V. Mann 1954 Influences of sulfur amino acid deficiency on cholesterol metabolism. Metabolism, 3:16.

11. Fillios, L.C., S.B. Andrus, G.V. Mann, and F.J. Stare 1956 Experimental production of gross athersclerosis in the rat. J. Exp. Med., 104:539.

12. Fraser, F.C., H. Kalter, B.E. Walker, and T.D. Fainstat 1954 The experimental production of cleft palate with cortisone and other hormones. J. Cell. Comp. Physiol., 43, suppl. 1:237.

13. Friedman, M., R.H. Rosenman, and V. Carrol 1958 Changes in the serum cholesterol and blood clotting time in men subjected to cyclic variation of occupational stress. Circ., 17:852.

14. Gero, S., J. Gergely, L. Jakab, J. Szekely, S. Virag, K. Farkas, and A. Czuppon 1961 Inhibition of cholesterol atherosclerosis by immunisation with B-lipoprotein. Lancet, Jan.-June:1119 (3rd Med. Clin. Budapest Univ.).

15. Gofman, J.W., F. Lindgren, H. Elliott, W. Mantz, J. Hewitt, B. Strisower, and V. Herring 1950 The role of lipids and lipoprotein in atherosclerosis. Science, 111:166.

16. Grundy, S.M. and A.C. Griffen 1959 Relationship of periodic mental stress to serum lipoprotein and cholesterol levels. J. Am. Med. Assoc., 171:1794.

17. Hartel, A. and G. Hartel 1960 Experimental study of teratogenic effect of emotional stress in rats. Science, 132:1483.

18. Hill, C.H. 1961 Dietary protein levels as they affect blood citric acid levels of chicks subjected to certain stresses. Poultry Sci., 40:762.

19. Hurder, W.P. and A.F. Sanders 1953 Audiogenic seizure and the adrenal cortex. Science, 117:324.

20. Homburger, F. and P. Bernfeld 1958 Lipoproteins: Methods and Clinical Significance. S. Karger, New York, New York.

21. Ingle, D.J. 1956 Some questions relating to the role of the adrenal cortex in the etiology of disease. IN: Stress by H. Selye, MD Publications, Inc., New York, New York.

22. Jones, R.J. and S. Huffman 1956 Chronic effect of dietary protein on hypercholesteremia in the rat. Proc. Soc. Exptl. Biol. Med., 93:518.

23. Jurtshuk, P. Jr., A.S. Wiltman, and A.M. Sackler 1959 Biochemical responses of rats to auditory stress. Science, 129:1424.

24. Katz, L.N., J. Stamler, and R. Pick 1958 Nutrition and Atherosclerosis. Lea and Febiger, Philadelphia, Pa.

25. Kuhl, W.J. Jr., E.M. Beck, H. Gershberg, E. Street, and E.P. Ralli 1955 Effect of cold water stress on blood and urine constituents in 55 normal male subjects. Metabolism, 4:143.

26. Levine, S. 1957 Infantile experience and resistance to physiological stress. Science, 126:405.

27. Mann, G.V., S.B. Andrus, A. McNally, and F.J. Stare 1953 Experimental atherosclerosis in Cebus monkeys. J. Exptl. Med., 98:195.

28. Millen, J.W. and D.H.M. Woollam 1957 Influence of cortisone on teratogenic effects of hypervitaminosis A. Brit. Med. J., 11:196.

29. Moyer, A.W., D. Kritchevsky, J.B. Logan, and H.R. Cox 1956 Dietary protein and serum cholesterol in rats. Proc. Soc. Exptl. Biol. Med., 92:736.

30. Nath, N., A.E. Harper, and C.A. Elvehjem 1958 Dietary protein and serum cholesterol. Arch. Biochem. Biophys., 77:234.

31. Nishida, T., A. Ueno, and F.A. Kummerow 1960 Effect of dietary protein on the metabolism of sodium acetate-1-C^{14} in chicks. J. Nutrition, 71:379.

32. Nishida, T., A. Ueno, and F.A. Kummerow 1960 Metabolism of cholesterol-4-C^{14} in bile duct cannulated chicks and rats. Circ. Res., 8:742.

33. Rama Rao, P.B., V. Chalam Metta, H.W. Norton, and B.C. Johnson 1960 The amino acid composition and nutritive value of proteins. III The total protein and the non-essential amino nitrogen requirement. J. Nutrition, 71:361.

34. Rasmussen, A.F. Jr., J.T. Marsh, and N.Q. Brill 1957 Increased susceptibility to herpes simplex in mice subjected to avoidance-learning stress or restraint. Proc. Soc. Exptl. Biol. Med., 96:183.

35. Selye, Hans 1950 The Physiology and Pathology of Exposure to Stress. ACTA, Inc., Medical Publishers, Montreal, Canada.

36. Uhley, H.N. and M. Friedman 1959 Blood lipids, clotting and coronary atherosclerosis in rats exposed to a particular form of stress. Am. J. Physiol., 197:396.

37. Wertlake, P.T., A.A. Wilcox, M.L. Haley, and J.E. Peterson 1958 Relationship of mental and emotional stress to serum cholesterol levels. Proc. Soc. Exptl. Biol. Med., 97:163.

38. Wistar, R. Jr., and W.H. Hildemann 1960 Effect of stress on skin transplantation immunity in mice. Science., 131:159.

39. Woodward, G.E., and E.G. Fry 1932 The determination of blood glutathione. J. Biol. Chem., 97:465.