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# THE EFFECT OF STRESS ON THE FORMATION OF TISSUE LIPID FROM DIETARY PROTEIN

Frederick H. Radke, Herman De Haas[1] and Sally Jacobs[2]

The effects of stress on an animal can be studied by many experimental techniques. All techniques are designed to seek measurable differences between stressed and unstressed animals and to determine by experiment or implication if the differences found have a deleterious effect on the animal.

There is no doubt that space travel is a stress and that this stress has an effect on the human beings who travel in space. Whether the stress of fatigue and monotony on a rat will produce the same effect as long-time space travel on the human needs to be tested as the effects are found.

In these laboratories considerable experience has been gained in measuring the carcass composition of rats, in measuring cholesterol levels of blood serum and tissues, and in feeding radioactive protein to rats in order to determine its metabolic fate. These techniques, together with the stress which was applied by placing the rat in a rotating drum in a black box, were used to study the effects of stress.

There are thousands of research papers on cholesterol levels in serum and tissues. A summarization of the authors' evaluation of these is available (1,2).[3] Since cholesterol is found in all tissues and is especially concentrated in the brain, variations in its concentration in the tissues with stress need to be evaluated.

## MATERIALS AND METHODS

### Stressing Drum

A smooth metal drum with a 24-inch diameter was housed in a wooden box (figure 1).[4] The edge of the drum was covered with rubber and flush with the box as the drum rotated. Screened vents provided fresh air but little light. The drum was rotated by a small electric motor (a capacitor-reducer motor and a 3.75 microfarad condenser) which was connected to the drum by two sprocket wheels connected by a chain. The drum was rotated one-half of the time, alternating random-

[1] Head and Associate Professor, respectively, Department of Biochemistry.

[2] Technician, School of Home Economics.

[3] Literature cited, page 9.

[4] The means of operation of the drum and the building of the device was done by Chase Langmaid III. Many alterations were required before the rats could withstand the rigors of the rotating drum.

length intervals of operation and rest. No interval exceeded four minutes. The motor was operated by a micro-switch (a variable interval timer which operated at a speed of one millimeter/second) which was programmed by passing a plastic tape with alternating holes and solid portions through the switch. The interval pattern was repeated eight times during the forty-eight hour stress period.

**Animals**

Male rats (CFN strain) were purchased from Carworth Farms (New City, Rockland County, New York). The group weight was $59 \pm 0.5$ grams when the first pair was started in the rotating drum. Six rats were rotated in the drum in pairs while six rats remained in the cages as controls. The stressed rats were rotated in pairs two days at a time and rested for four days following this. The limit of the rats' endurance had been found previously by sacrificing several rats due to over-stress. The stress program was continued for 6 to 8 weeks.

**Diet**

The following diets were fed *ad lib.*

| A | | B | |
|---|---|---|---|
| | % of Diet | | % of Diet |
| Casein[1] | 20 | Rat Protein[2] | 28 |
| Sucrose | 64.5 | Sucrose | 56.5 |
| Corn Oil[3] | 5 | Corn Oil[3] | 5 |
| Alphacel[4] | 4 | Alphacel[4] | 4 |
| Salt Mix[5] | 4 | Salt Mix[5] | 4 |
| Vitamin Mix[6] | 2 | Vitamin Mix[6] | 2 |
| DL-Methionine[7] | 0.3 | DL-Methionine[7] | 0.3 |
| Choline Chloride[7] | 0.2 | Choline Chloride[7] | 0.2 |

[1] Nutritional Biochemicals Vitamin Free Casein
[2] Prepared in this laboratory. 72% protein
[3] Mazola
[4] Nutritional Biochemicals refined cellulose
[5] Jones-Foster Salt Mix from Nutritional Biochemicals
[6] Composition of vitamin mix in reference 3, page 9.
[7] Nutritional Biochemicals Corporation

Diet A was fed throughout the stressing period. Diet B was fed during a four-day period immediately following the final stress period for each pair of rats used in the tracer experiments (experiment II).

The rat protein containing $C^{14}$ was prepared by feeding protein from the photosynthetic bacterial species *Rhodospirillum rubrum* which had been grown on a medium containing $C^{14}O_2$ as a carbon source, and extracting the tissues from these rats with alcohol followed by ether (3). The rat tissue (containing 12.9% bone) thus prepared had an activity

of 1,600,000 counts per minute per gram and Diet B had an activity of 392,000 counts per minute per gram. The rats consumed an average of 83 grams of the diet or a total count per minute of 32,540,000 (experiment II).

**Tissue Collection and Preparation**

The rats were killed by decapitation. Blood samples were collected and the serum obtained from it was immediately frozen and stored at -20°C until analysis. The following tissues were excised: liver, heart, epididymal fat pads, gastrocnemius muscle and brain. Weighing was done on a Mettler B-6 balance and drying in a vacuum drying oven at 90°

**Lipid Determinations**

The dried tissue was ground in a micro-Wiley mill and extracted with ether in a micro-Soxhlet apparatus. After being weighed, the extracted lipid was used for lipid determinations and, where appropriate, $C^{14}$-activity determinations. Radioactivity determinations were done in triplicate.

**Radioactivity Counting System**

$C^{14}$-activity was assayed in a system consisting of a Tracerlab FD-1 Flow Counter, a P-31 Geiger Preamplifier, an SC-100 Multimatic Sample Changer, an SC-71 Compu-matic Scaler and an SC-87A Auto-Printer. Gas used in the flow counter was a mixture of 98.7% helium and 1.3% butane.

**Cholesterol Determination**

A modified Zlatkis method was used (2,4) for cholesterol determinations. Lipid extracts were diluted to 10 ml with 2:2:1 ethanol-chloroform-ether. Aliquots of the following volumes were done in triplicate: liver 50 λ, fat pads 10 λ, heart 250 λ, brain 25 λ. The muscle tissue could not be run by this method due to endogenous color. The aliquots were evaporated to dryness in a boiling water bath. Two ml of glacial acetic acid were added. A color reagent (8 ml of solution of 2.5 g of $FeCl_3 \cdot 6H_2O$ per 100 ml $H_3PO_4$ were diluted to 100 ml with concentrated $H_2SO_4$) was made up immediately beforehand and 1.5 ml added to each tube. The contents were mixed and read, after one-half hour at room temperature, in a Beckman DU Spectrophotometer at 560 mu with a slit width of 0.04.

For serum analysis, 0.1 ml serum was pipetted into a centrifuge tube, and 5.5 ml of a 1:1 mixture of 95% ethanol-ether were added.

After mixing, the contents were centrifuged in stoppered centrifuge tubes at 4,750 rpm for 20 minutes. Triplicate aliquots of 1.0 ml were evaporated in a boiling water bath and the procedure for adding acetic acid and color reagent was followed as above.

### $CO_2$ Collection

Carbon dioxide was collected by placing the rats in Delmar glass metabolism cages. Air was brought into the cage through Drierite and Ascarite to remove water and $CO_2$, and the exhaled $CO_2$ was collected in 1 N NaOH as it passed out of the cage. The same NaOH was used during all the hours specified for a single rat. The NaOH-$Na_2CO_3$ was then plated in triplicate on planchets and the counts per minute from the $C^{14}$ determined after evaporation to dryness.

### Feeding

The growth rates of the stressed rats and the controls were equalized by limiting the amount of food provided for the controls. The stressed rats were allowed to eat all they wanted. The first two experimental groups of rats were run for six weeks and averaged $273 \pm 6.8$ grams at the time of decapitation. The second two experimental groups were run similarly for six weeks. During the next two weeks, as each pair of stressed rats completed a stress period, they and a pair of control animals were fed the radioactive protein diet for 100 hours. Rats were kept in the metabolism cages for the collection of $CO_2$ during hours 48-56, 72-80 and 96-100. They were sacrificed during hour 100 and weighed $312 \pm 9.7$ grams. Figure 2 shows the growth curves for the two series of rats.

## RESULTS

The fat contents of the heart, liver, brain, muscle and epididymal fat pads were not significantly different between stressed and unstressed rats. Low fat (heart), medium fat (brain), and high fat (epididymal fat pads) tissues were represented in table 1.

The moisture contents of the tissues also revealed no differences of note (table 2) except in the epididymal fat pads where the stressed rats had a higher moisture content ($P < .01$ and $P < .2$). The high fat content of the epididymal fat pads makes it an unusual tissue, and in two comparisons there were 140 and 150% as much moisture in the stressed rats as compared to the unstressed. It is doubtful that this difference is important in this very high fat tissue.

Tables 3, 4 and 5 were prepared from data taken on rats which had been fed radioactive rat protein after having received casein as a

source of dietary protein. The feeding of the radioactive protein was done in order to test for differences in protein metabolism due to stress.

Table 3 shows the $C^{14}$-activity found in the complete organ tissue of the heart, brain and liver. The $C^{14}$-activity was corrected to a common organ weight, rat weight and food consumption. The liver contained a higher $C^{14}$-activity in the stressed animals ($P<.10$).

The $C^{14}$-activity in the fat extracted from the heart, liver and muscle was significantly higher in the fat from the stressed rats (table 4). The $C^{14}$-activity level in the brain and fat pads was not significantly different in the stressed and unstressed animals. This greater conversion of $C^{14}$ from protein into fat in the three tissues reveals that there is a difference in the utilization of dietary protein when stress is applied. The complex metabolic pathways leading from protein to fat most likely finds acetyl coenzyme A, formed from protein utilization for energy, being utilized to form fatty acid molecules which are converted to fats. It is interesting to note that the tissues which are more active metabolically incorporate the greatest amount of $C^{14}$-activity, reflecting the greater turnover rates of fat in these tissues. The order of decreasing $C^{14}$-activity is liver>heart>muscle>brain>fat pads. The brain and fat pads both have a higher lipid content than the other tissues but the metabolic activity of the lipid is lower.

Table 5 indicates that the higher $C^{14}$-activity found in the $CO_2$ exhaled by the stressed rats was not significantly different from that of the unstressed rats. The $C^{14}$-activity of carbon dioxide represents the radioactive protein used for energy purposes.

The serum cholesterol levels in the rats which were exercised were significantly lower than in the rats which were not exercised (table 6). Exercise also results in lower serum cholesterol levels in human beings. In space travel, diets must be designed which lead to low serum cholesterol levels and provision must be made for exercise.

Tables 7, 8 and 9 point out a difference in the cholesterol level in a very important tissue—the brain. The composition of the brain is more constant than most other tissues, and these highly significant differences in cholesterol levels indicate an effect of stress on the brain. Cholesterol is relatively unreactive metabolically once it is formed in a tissue like the brain. However, a difference in rates of metabolism had to lead to its greater formation in the stressed animals. This is in contrast to the lower serum cholesterol levels in the stressed animals.

In the case where only casein was fed for six weeks, the liver had a higher cholesterol level but in the case where rat protein was also fed the reverse was noted. The liver is the most active tissue metabolically, but does not have nearly as high a cholesterol level as the brain.

In this laboratory in other experiments with hundreds of rats of many ages and dietary conditions, the brain cholesterol level varied from 1.35 to 2.14% on a wet weight basis. On a dry weight basis this is 4.87 to 9.30%. The rats in the present experiments received the same diets, hence, the difference was due to stress not diet. One experiment showed 1.72% brain cholesterol in the stressed group and 1.39% in the unstressed group. The corresponding dry weight levels of cholesterol are 7.81 and 6.31% A second experiment resulted in 2.49% for stressed and 1.90% for unstressed rats on a wet weight basis and 10.8 and 8.28% on a dry weight basis.

The fact that a higher cholesterol level does occur in the brain due to stress, 24% more in one case and 31% more in the other, merits further investigation. One facet which needs investigation is the effect of the extra cholesterol on brain metabolism. Another is the effect on brain function in terms of behavior and reaction.

The adrenal cortex and the testes which utilize cholesterol for the synthesis of steroid hormones need to be studied. These endocrine glands are known to produce higher hormone levels at times of stress. Whether or not changes in cholesterol levels as a result of stress influence the production of hormones by these tissues should be determined.

## SUMMARY

Rats were stressed in a black, enclosed, motor-driven drum by being rotated at random intervals during half of a forty-eight hour period. They were stressed for two out of every six days for six weeks, while receiving a casein diet. In a second experiment, after six weeks, the rats were fed $C^{14}$-labeled rat tissue protein for one hundred hours immediately after the last stress period and placed in glass-enclosed metabolism cages for specified hours during this period so that the exhaled carbon dioxide could be collected. Blood, heart, liver, brain, muscle and epididymal fat pads were removed for analysis.

The fat content of the tissues was not significantly different between the stressed and unstressed rats.

The epididiymal fat pad of the stressed rats had a higher moisture content than those of the unstressed.

The $C^{14}$-activity of the fat extracted from the heart, liver and muscle of the stressed rats was significantly higher than that of the unstressed rats.

$C^{14}O_2$-activity of exhaled air from stressed and unstressed rats was not significantly different.

Serum cholesterol levels of the stressed rats were significantly lower than those of the unstressed rats.

The cholesterol level in the brains of the stressed rats was significantly higher than in the brains of the unstressed rats.

## LITERATURE CITED

1. Radke, F. H. and H. De Haas. Maine Farm Research *12*, 8 (1964).
2. Radke, F. H., H De Haas, E. K. Gabrielson and M. E. Norburg. Maine Agricultural Experiment Station Technical Bulletin (Manuscript Prepared).
3. Radke, F. H., M E Norburg and E. K. Gabrielson. Maine Agricultural Experiment Station Technical Bulletin T 1, 1962.
4. Zlatkis, A., B. Zak and A. J. Boyle, J. Lab. Clin. Med. *41*, 486 (1953).

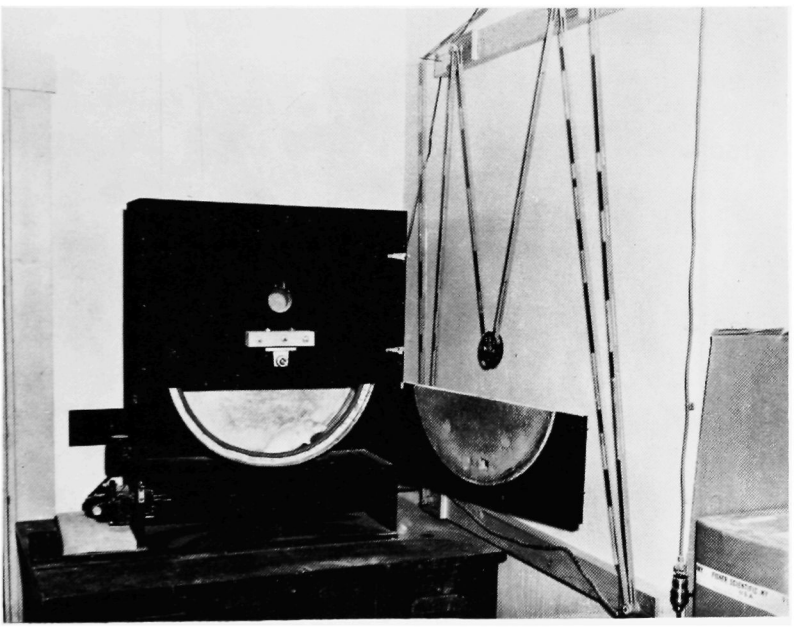

Figure 1. Rotating black drum with programming device.

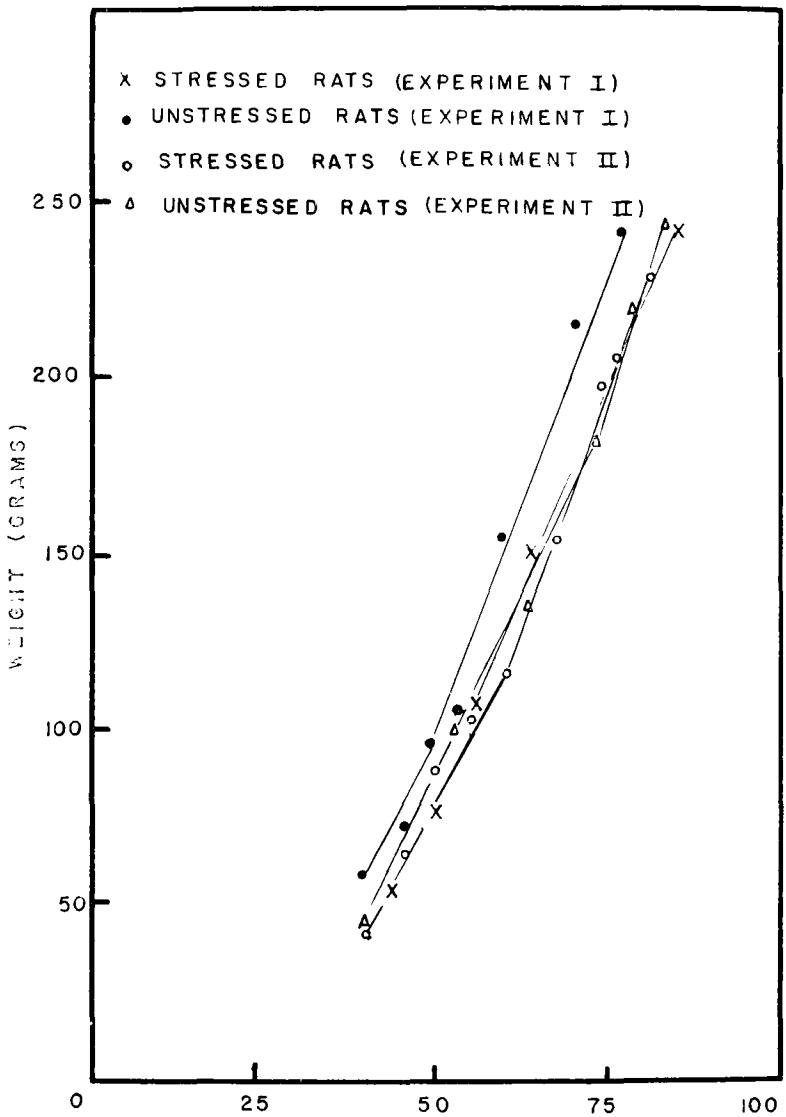


Figure 2. Change in average weight of stressed and unstressed rats versus time.

Table 1

Fat content of tissues of stressed and unstressed rats

| Tissue | Experiment[1] | % Fat (Dry Weight) Stressed group[2] | Unstressed group[2] |
|---|---|---|---|
| Heart | I | 8.6±0.7[3] | 6.8±0.8 |
| | II | 14.0±2.0 | 14.0±3.2 |
| Liver | I | 11.0±2.2 | 11.8±1.0 |
| | II | 10.0±0.7 | 14.0±1.7 |
| Brain | I | 30.4±0.3 | 32.5±1.7 |
| | II | 37.0±0.8 | 36.0±2.0 |
| Muscle | I | —— | —— |
| | II | 17.3±1.7 | 23.2±2.6 |
| Epididymal Fat Pads | I | 95.2±0.5 | 96.4±0.4 |
| | II | 87.3±4.9 | 82.2±1.7 |

[1] Casein was fed for 6 weeks in experiment I. Casein was fed for 6 to 8 weeks followed by the feeding of radioactive rat protein for 100 hours in experiment II.
[2] Six rats per group.
[3] Standard errors of the mean are included.

Table 2

Water content of tissues of stressed and unstressed rats

| Tissue | Experiment[1] | % Water Stressed group[2] | Unstressed group[2] |
|---|---|---|---|
| Heart | I | 76.8±0.2[3] | 76.1±0.1 |
| | II | 75.8±0.4 | 75.0±0.8 |
| Liver | I | 70.2±0.2 | 70.5±0.4 |
| | II | 69.2±0.9 | 68.4±0.6 |
| Brain | I | 77.8±0.1 | 77.6±0.2 |
| | II | 77.3±0.2 | 77.0±0.2 |
| Muscle | I | —— | —— |
| | II | 72.7±0.8 | 69.8±1.5 |
| Epididymal Fat Pads | I | 18.0±1.4 | 12.4±1.0 |
| | II | 14.1±1.8 | 10.0±1.4 |

[1] Casein was fed for 6 weeks in experiment I. Casein was fed for 6 to 8 weeks followed by the feeding of radioactive rat protein for 100 hours in experiment II.
[2] Six rats per group.
[3] Standard errors of the mean are included.

Table 3

$C^{14}$-activity of protein origin in tissues of stressed and unstressed rats

| Tissue | Counts per minute per total organ weight per gram of rat per 100 grams of food consumed: Stressed group[1] | Unstressed group[1] |
|---|---|---|
| Heart | 9.8±1.8[2] | 7.0±0.8 |
| Brain | 10.7±1.7 | 11.2±0.9 |
| Liver | 266±24.0 | 200±21.7 |

[1] Six rats per group.
[2] Standard errors of the mean are included.

Table 4

$C^{14}$-activity of protein origin in the fat of tissues of stressed and unstressed rats

| Tissue | Counts per minute per milligram of fat per 100 gram rat per 100 grams of diet consumed | |
|---|---|---|
| | Stressed group[1] | Unstressed group[1] |
| Heart | 26 ±3.8[2] | 16.5±1.2[3a] |
| Liver | 68±4.0 | 33.7±4.2[3b] |
| Brain | 7.6 ±0.7 | 7.5±0.9 |
| Muscle | 14.4±3.1 | 6.6 ±0.7[3a] |
| Fat Pads | 7.4±1.7 | 5.2 ±0.9 |

[1] Six rats per group.
[2] Standard errors of the mean are included.
[3] Difference between stressed and unstressed.
a. $P < .05$
b. $P < .001$

Table 5

$C^{14}$-activity of protein origin in carbon dioxide from stressed and unstressed rats

| | Stressed group[1] | Unstressed group[1] |
|---|---|---|
| Counts per minute per gram of rat per gram of food | 472±42[2] | 400±38 |

[1] Six rats per group.
[2] Standard errors of the mean are included.

Table 6

Serum cholesterol levels in stressed and unstressed rats

| Experiment[1] | Serum cholesterol levels —mg% | |
|---|---|---|
| | Stressed group[2] | Unstressed group[2] |
| I | 121±7[3] | 151±7[4a] |
| II | 115±9 | 174±11[4b] |

[1] Casein was fed for 6 weeks in experiment I. Casein was fed for 6 to 8 weeks followed by the feeding of radioactive rat protein for 100 hours in experiment II.
[2] Six rats per group.
[3] Standard errors of the mean are included.
[4] Difference between stressed and unstressed.
a. $P < .01$
b. $P < .005$

Table 7

Cholesterol levels in tissue fat of stressed and unstressed rats

| Tissue | Experiment[1] | Mg cholesterol per gram of fat | |
|---|---|---|---|
| | | Stressed group[2] | Unstressed group[2] |
| Heart | I | 92±9[3] | 112±32 |
| Liver | I | 104±9 | 79±8 |
| | II | 69±4 | 78±3 |
| Brain | I | 257±4 | 194±13[4a] |
| | II | 292±9 | 230±17[4b] |
| Fat Pads | I | 25±0.4 | 28±1.7 |

[1] Casein was fed for 6 weeks in experiment I. Casein was fed for 6 to 8 weeks followed by the feeding of radioactive rat protein for 100 hours in experiment II.
[2] Six rats per group.
Standard errors of the mean are included.
[4] Difference between stressed and unstressed.
a. $P < .01$
b. $P < .005$

Table 8

Cholesterol levels in the dry tissue of stressed and unstressed rats

| Tissue | Experiment[1] | Mg cholesterol per gram of dry tissue | |
|---|---|---|---|
| | | Stressed group[2] | Unstressed group[2] |
| Heart | I | 7.9±0.8[3] | 7.6±2.2 |
| Liver | I | 11.4±1.0 | 9.3±0.9 |
| | II | 6.9±0.4 | 10.9±0.4[4a] |
| Brain | I | 78.1±1.2 | 63.1±4.2[4b] |
| | II | 108.0±3.3 | 82.8±6.1[4c] |
| Fat Pads | I | 23.8±0.4 | 27.0±1.6 |

[1] Casein was fed for 6 weeks in experiment I. Casein was fed for 6 to 8 weeks followed by the feeding of radioactive rat protein for 100 hours in experiment II.
[2] Six rats per group.
[3] Standard errors of the mean are included.
[4] Difference between stressed and unstressed.
a. $P < .001$
b. $P < .01$
c. $P < .005$

Table 9

Cholesterol levels in the tissue of stressed and unstressed rats

| Tissue | Experiment[1] | Mg cholesterol per gram of wet tissue | |
|---|---|---|---|
| | | Stressed group[2] | Unstressed group[2] |
| Heart | I | 1.82±0.18[3] | 1.83±0.52 |
| Liver | I | 3.43±0.30 | 2.80±0.28 |
| | II | 2.14±0.12 | 3.49±0.13[4a] |
| Brain | I | 17.19±0.26 | 13.87±0.93[4b] |
| | II | 24.85±0.76 | 19.04±1.35[4c] |
| Fat Pads | I | 19.52±0.33 | 23.75±1.44[4d] |

[1] Casein was fed for 6 weeks in experiment I. Casein was fed for 6 to 8 weeks followed by the feeding of radioactive rat protein for 100 hours in experiment II.
[2] Six rats per group.
[3] Standard errors of the mean are included.
[4] Difference between stressed and unstressed.
a. $P < .001$
b. $P < .01$
c. $P < .005$
d. $P < .025$