**PAPER ELECTROPHORESIS OF RAT SERUM AND MUSCLE PROTEINS
DURING HYPOTHERMIA AND COLD ACCLIMATIZATION**

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the Faculty of the Graduate School
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of the Requirements for the Degree
Master of Arts**

**by
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The undersigned, appointed by the Dean of the Graduate Faculty, have
examined a thesis entitled PAPER ELECTROPHORESIS OF RAT SERUM AND
MUSCLE PROTEINS DURING HYPOTHERMIA AND COLD ACCLIMATIZATION

presented by Jimmie L. Shields

a candidate for the degree of Master of Arts

and hereby certify that in their opinion it is worthy of acceptance.

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INTRODUCTION

Exposure of animals and man to low environmental temperature causes certain definite alterations in the physiological state. These changes fall into two classifications. First, the acute adjustments which the organism makes under the stimulus of the initial, stressful situation, and finally slower and longer-lasting alterations occur when the animals are maintained for long periods of time in the cold environment.

Many terms and phrases have found their way into the literature describing the longer-lasting adjustments in animals. Adaptation, according to Burton and Edholm (1955) is defined as the adjustments which occur after several generations of living in a foreign environment. More recently, Carlson at the Federation Meeting (1958) in a Symposium on Acclimatization, defined the term acclimatization as "to render fit". He regarded adaptation and acclimatization as interchangeable terms. Such disagreement is understandable since it would be difficult to define a condition which is not completely understood. Indiscriminate use of the terms can lead to much confusion and, therefore, it seems expedient to define some of the chronic effects of continued cold exposure. Acclimatization in the present study is defined as the adjustments made by an organism when exposed to a radical change in its environment, which enable the organism to survive continued exposure.

When cold stress is of sufficient severity, the organism is unable to withstand the initial shock of its onset. Hypothermia ensues and if the stressor is not removed, death follows due to cardio-vascular dysfunction (Swan, 1953, Fleming, 1954). However, if a less severe cold stress is applied, the temperature regulating mechanism is able to stimulate heat production in the animal which will allow it to exist without any appreciable change in body temperature.

Disagreement still exists regarding the exact nature and cause of this increased heat production, although the important role of the thyroid hormone is generally indicated (Ring, 1939, Sellers, 1950). The increased metabolism of the liver during cold exposure has been cited recently as possibly responsible for much of the overall increased metabolic response (Weiss, 1954, Weiss, 1957).

The present study is designed to follow the course of the electrophoretic pattern of the serum proteins and water extractable muscle proteins during cold acclimatization. Body weight changes and body temperatures were determined also. Since the liver is considered the predominant source of the serum proteins, it may be interesting to note any changes that occur in these indices of liver function. Attention was given also to differences in the electrophoretic patterns of serum proteins after short term exposure to acute cold stress, and to sex differences during the acclimatization period. The importance of the thyroid in acclimatization has prompted the use of thyroid extract as a supplement in the diet of some of the experimental animals. This was done in an attempt to simulate a more severe stress during the course of the experiment.

HISTORICAL REVIEW

The realization that conditions of environmental temperature have an effect on the metabolism of organisms apparently dates back as far as Lavoisier in the 18th century (Weiss, 1957). Benedict and MacLeod (1929) reported that albino rats kept at 21°C. for one day prior to study showed elevated heat production over controls maintained at 28°C.

Short term cold torpor became of interest when it was discovered that various animals were able to survive a period of hypothermia with no apparent ill effects. In an article by Britton (1930), mention is made of work by Hunter, in 1837, who found that the body temperature of the eel could be reduced to approximately 0°C., and that although the eel first appeared to be dead, recovery took place when the temperature of the surrounding water was raised. Also Ross, in 1898, repeatedly froze and revived caterpillars, Larva rossi, reaching a minimal temperature of -42°C. (Britton, 1930). Britton's work had shown that the heartbeat of various fish stiffly frozen was completely stopped and upon rewarming normal rhythm was recovered and other metabolic and general life processes returned to normal.

The influence of low temperature on true homeothermic animals was apparently first investigated systematically in the laboratory of Walther (1862). Experimenting with immobilized, non-anesthetized rabbits, he found that cooling to a rectal temperature of 20°C.

abolished the capacity for spontaneous recovery of normal body heat, and that artificial warming was necessary and successful in complete restoration of the normal state. Cooling below 20°C., he believed, inevitably led to death.

Britton (1922, 1930) tested the various reflexes under hypothermia in non-hibernating animals and concluded that, in many ways, the effects of gradual cooling bear a strong similarity to those induced by anesthesia. Interest in hypothermia was greatly stimulated by this suggestion of a substitute or supplement to surgical anesthesia. The greatest danger in this method seemed to be the incidence of cardiac failure.

Lethal body temperatures in several species of infant and adult mammals were established by Fairfield (1948) and Adolph (1948, 1951). These authors demonstrated that infant animals were able to survive more profound levels of hypothermia than adults of the same species.

The clinical applications of hypothermia assumed greatest dimensions with the work of Bigelow, Lindsay and Greenwood (1950). With moderate hypothermia from 26° to 28°C., total circulatory interruption, resulting in a bloodless heart, was possible for about ten minutes. Experimental work with hypothermia was greatly stimulated by the realization that new methods in cardiac surgery were dependent on the ability of an animal to withstand the stress of reduced body temperature.

The main handicap to surgery was the short time which the circulation could be interrupted. Lower levels of hypothermia could lengthen this time, but at these lower levels fatal ventricular fibrillation was a problem. It was established that it was not hypoxia, but

rather the accumulation of carbon dioxide in the blood which led to a decreased arterial pH (acidosis). This acidosis was regarded as responsible for the ventricular fibrillation encountered in the hypothermic animals (Adolph, 1948, Swan, Zeavin, Holmes and Montgomery, 1953, Fleming, 1954).

Attempts to remove the excess carbon dioxide of the blood by forced or hyperventilation resulted in more profound levels of hypothermia and a greatly reduced incidence of ventricular fibrillation (Osborn, 1953, Fleming, 1954).

The next step was the achievement of sustained cardiac arrest. Niazi and Lewis (1954) accomplished this with rats by careful methods of cooling and artificial respiration and reported sustained cessation of heartbeat for periods of two to three hours. Rectal temperatures in these experiments reached low points between -4.0°C . and 8.5°C .

In subsequent publications, similar results were reported with dogs and monkeys (Niazi and Lewis, 1954 and 1957). These animals survived for indefinite periods of time (i.e. many weeks). It had previously been held that asystole was lethal for adult homeotherms if sustained as long as ten seconds (Fairfield, 1948, Adolph, 1948).

The work of Segar, Riley and Barila (1956) furnishes information on the chemical changes during acute cold stress. Rectal temperatures of dogs were reduced to 20°C . by packing in an ice bath and artificial ventilation was used during the resulting hypothermia. Serum and urine samples were collected. The urine flow in cubic centimeters per minute, urine pH, urine sodium and urine chlorides increased upon cooling the animals. These values then decreased to normal or slightly

below upon rewarming. Total urine carbon dioxide showed a slight drop during the entire process. Urinary ammonia decreased from 48.6 mg. per cent to 2.7 mg. per cent upon cooling, and approached normal values upon rewarming. Potassium levels dropped and remained low upon rewarming. Creatinine levels also dropped and then showed a very slight increase upon rewarming.

The most impressive chemical changes in the serum are decreased potassium levels and an increased pH. The latter was explained as a mild respiratory alkalosis due to the forced ventilation. The increase was from pH 7.43 to 7.52. Evidence of respiratory alkalosis has been reported whenever forced or hyperventilation is employed (Osborn, 1953, Fleming, 1954).

Since potassium levels in both serum and urine decrease, this ion is apparently moving into the intracellular spaces. The details of this shift of potassium into and out of the intracellular space, which occurs with pH changes, are poorly understood. In respiratory acidosis, potassium enters the serum. MacKay (1947) suggests the liver as a possible source of this extra potassium, since hyperkalemia did not occur in hepatectomized animals. Serum levels of carbon dioxide, sodium, chlorides, and creatinine showed no significant change. Hematocrit values dropped slightly.

The work of Swan, Zeavin, Holmes and Montgomery (1953) previously mentioned in this manuscript, also indicated chemical changes in serum and urine of dogs cooled to 20° to 25°C. deep rectal temperature. Sodium and chlorides showed no significant change. Serum phosphorus and hematocrits showed minor drops. Serum potassium decreased sharply.

A mild respiratory alkalosis occurred as a result of the hyperventilation employed. The plasma protein was reported as showing a very slight decrease in concentration. Blood volume changes were not statistically significant. Urine flow was reported as being lowered with no significant changes in electrolyte excretion.

Masacchea and Sievers (1956) have reported plasma protein concentrations after long and short term cold torpor as showing no change in the Chrysemys picta, a fresh water turtle commonly subjected to environmental temperature variations within a range of 0° to 35°C.

This review will now be directed to include studies of less severe environmental temperature, but with continued exposure for relatively long periods of time so that acclimatization occurs.

A huge volume of work appears in the literature dealing with the metabolic response to a cold environment. It has been shown by several investigators that during prolonged exposure to cold environmental temperatures, mammals undergo certain gradual changes which increase their resistance to cold. For example, Horvath, Hitchcock and Hartman (1938) showed that three days exposure to 4°C. temperature caused a 22 per cent rise in the basal metabolic rate of albino rats. Furthermore, they reported that colonic temperatures of normal and unilaterally adrenalectomized rats remained practically unchanged at temperatures of 4°C. and 29°C.

Ring (1939) observed that living for three weeks or more at 0°C. to 5°C. produced an average elevation in metabolism of 20 per cent in rats. Ring reported also a slight accompanying rise in the body temperatures. Thyroidectomy in these animals indicated that the

increase in metabolism is brought about largely, if not entirely, by the thyroid gland.

However, MacBeth and Noble (1949) showed that if thyroidectomy were performed after the animals had been acclimatized to low temperatures, subsequent exposure was marked by an increase in metabolism just as with an intact thyroid. Although this level was not as high as in the normal animals, the absolute rise in metabolism was practically the same. These facts led to the belief that the thyroid gland was not absolutely necessary for the acclimatization response of increased metabolism.

Leblond and Gross (1943), Sellers and You (1950), and Sellers, You and Thomas (1951) compared the metabolic response by exposure to cold immediately after thyroidectomy and two weeks post-operative. The increase in metabolism and also the time of survival was much lower in rats which were exposed to low temperatures two weeks after thyroidectomy. This led to the present concept that it is the existence of the thyroid hormone at the tissue level which is responsible for an initial increase in oxygen consumption. Thus it would appear that the thyroid gland is necessary in the early stages of acclimatization to a cold environment. The presence of the thyroid gland is not necessary after acclimatization is achieved although hyperplasia of the thyroid gland is present during acclimatization (Burton and Edholm, 1955).

Sellers and You (1950) measured the oxygen consumption of the cold exposed animals both at 1.5°C. and at 30°C. A rise in metabolism measured at 1.5°C. occurred immediately after exposure. The rise reached its maximum in two weeks when measured at 30°C. These high

levels were maintained for the entire three months of cold exposure.

The metabolic rate was greatly reduced when the animals were anesthetized with sodium pentobarbital and measured at 1.5°C . This implies that muscular activity is an important factor in the observed metabolic increase in animals exposed to cold. However, increased muscle activity did not appear to be responsible for the raised metabolic rate as measured at 30°C . since anesthesia did not cause a great lowering of the rate when measured at that temperature.

Krog, Monson and Irving (1955) have shown a 60 per cent increase in the metabolic rate of albino rats conditioned to cold. Rats previously conditioned to 5°C . for six weeks were able to maintain their body temperatures for two and one-half hours at -10°C ., but in non-acclimatized animals the corresponding tolerance level was 10°C . The authors consider this difference in the critical temperatures as another criterion of acclimatization.

The metabolic response to cold is due, at least in part, to increased muscular activity in the form of shivering or increased muscle tone. Sellers, Scott and Thomas (1954) measured the electrical activity of skeletal muscles of rats acclimatized to cold and found an increase over controls. The increase was not great enough to explain the over-all observed metabolic increase, however. This led these workers to suggest that the muscular activity in the acclimatized rat is a "second line of defense" in the maintenance of body temperature on exposure to cold. Apparently muscular activity provides a sufficient measure of heat production until the visceral tissues can assume the greater role when the animal has acclimatized.

Burton and Edholm (1955) have stated that animals placed in a cold environment increase their food intake 60 to 70 per cent soon after exposure. They suggest that the amount of muscular activity observed may not be adequate to explain the increased food intake.

Dugal, quoted by Burton and Edholm (1955), has followed the weight changes in young rats exposed to cold. He reported a weight decrease during the initial exposure to cold. He refers to this weight loss as the stage of resistance. Next is a slow recovery of weight to the initial level, which he calls the state of acclimatization. Then growth continues at the pre-cold rate and he describes this as the state of adaption.

That adrenal weight increases with prolonged exposure to cold is agreed upon by many authors (Leblond and Gross, 1943, Morin, 1946, Schaeffer, 1946). Most workers believe this weight increase is associated with the adrenal cortices, but Morin (1946) and Schaeffer (1946) consider that the medulla is also enlarged.

Dugal and Thérien (1947) have investigated the ascorbic acid levels of various tissues in the guinea pig. Their work shows an increase in ascorbic acid levels of liver, spleen, and testes, which occur after an initial decrease during the first few hours of exposure. The increase is gradual and continues for several weeks. The tissue accumulation is not an occurrence of diminished urine excretion of the agent, since the amount of ascorbic acid in the urine is also increased in the cold.

Mention has been made previously in this manuscript of the inference of non-muscular components in the elevation of metabolism by

the work of Sellers and You (1950) and Sellers, You and Thomas (1951). Hsieh and Carlson (1957) showed that thyroidectomized, cold-exposed rats reduce their caloric intake in spite of a continued requirement and capacity for heat production since their metabolic rates did not decrease initially, but after a few days. It was suggested that fat stores are being utilized to maintain the increased metabolic activity. It was further hypothesized that it is possibly the depletion of these fat stores that causes the terminal failure of thermoregulatory ability in the thyroidectomized rats rather than a direct effect of absence of the thyroid hormone (as advocated by Leblond and Gross, 1943, Sellers and You, 1950, and Sellers, You and Thomas, 1951).

Weiss (1954), studied the oxygen uptake of tissue slices from rats which had been exposed to 5°C. for ten to twenty-six days. Tissues of six week old rats showed increased oxygen consumption in every case except brain tissue. Liver exhibited the greatest increase and kidney the least. Six month old rats showed an increase in liver tissue only. Weiss interpreted this as signifying that older animals do not acclimatize as quickly.

In a subsequent publication, Weiss (1957) studied the effects of experimental hypothyroidism on oxygen uptake of tissue. His results suggest that only liver and heart tissue increase in oxygen consumption with cold exposure in the absence of the thyroid hormone. He maintains that thyroid hormone does play an important role in the regulation of oxygen consumption of selected tissues and in subsequent successful acclimatization of the animal to a cold environment. The time of exposure in this experiment was five days. Weiss believes that the

animal may be considered acclimatized at five days since this is the period which shows continuous elevation of tissue oxygen consumption in vitro.

Hannon (1958) also studied rat liver oxygen consumption in vitro after prolonged cold exposure. His study was designed to determine whether the duration of exposure affects the rate of liver respiration in vitro. Hannon's results show a maximum QO_2 at four weeks exposure, after which time the values decreased and approached a nearly normal level at twelve weeks in the cold. His work, therefore, confirms that of others (Weiss, 1954 and Sellers and You, 1951) concerning the response of liver metabolism, but contrary to the opinions of Weiss (1954, 1957), the duration of exposure is definitely critical as regards such a response.

Anaerobic glycolysis was reported as decreasing significantly and progressively with the length of sojourn in the cold environment. The reason for this increase is left open to suggestion by the investigator. Hannon (1958) likewise offered data that indicate that fasting for twenty-four to thirty hours before sacrifice has little or no effect on the in vitro metabolism of rat liver slices.

You and Sellers (1951) have reported liver hypertrophy, increased oxygen consumption in vitro, and increased succinoxidase activity of liver slices after exposure of rats to low temperatures and subsequent acclimatization.

The prominence of the liver in maintaining increased metabolism in the cold is indeed interesting, but not surprising. It has been suggested that the liver is responsible for about 20 per cent of the

resting metabolism of the whole animal (Huston and Martin, 1954).

Such increased activity in the cold might then be expected to have an influence on the rate of production of serum proteins from that organ. The nature of serum protein changes due to continued cold exposure is somewhat unsettled.

Heroux (1958) has reported the effect of four week acclimatization to cold on organ weights. He found no changes in brain, genitals and lungs. Muscle, pelt, fat, skeleton, spleen, and thymus underwent a reduction in growth. Tissues which showed hypertrophy were liver, intestine, kidney, heart and adrenals.

The increase in adrenal, thyroid, and pituitary gland weights has been reported by others (Catz, Geiger, Thomas and El Rawi, 1954, Leblond and Gross, 1943).

Heroux and Gridgeman (1958) performed a second study with reference to cold acclimatization giving special attention to the weight and composition of skeletal muscles. After eight weeks at 6°C., female white rats showed a smaller muscle mass than controls of the same age maintained at 30°C. Water and protein determinations in soleus and gastrocnemius muscles, as well as in the whole muscle mass, revealed that the reduction in the muscle mass after cold acclimatization is a real tissue difference due to a reduced protein deposition and not only a water content difference. Red and white muscle fibers were equally affected as shown by comparison of percentage changes in soleus (nearly 100 per cent red fibers) and gastrocnemius (60 to 75 per cent white fibers).

The serum proteins of rabbits were studied by Waugh (1952) after

a twenty day exposure to temperatures ranging from -9°C . to 4°C . Determinations were made by salt fraction methods and by electrophoretic separation. No significant changes were found and the electrophoretic data were not included in the results. Apparently this had been only a method of checking the results of fractionation.

Sutherland and Campbell (1956) studied changes in blood clotting and electrophoretic properties of rabbit plasma after continuous exposure to 4°C . for two months. Male animals were used, and clipping of the fur was employed to intensify the stress. They report an increase in platelet and erythrocyte counts and in hematocrit. Blood and plasma clotting times were increased. From the electrophoretic analyses, cold acclimatization resulted in a significant decrease in albumin and a significant increase in beta-globulin and fibrinogen fractions. The greatest change was seen in the fibrinogen increase as determined by salt fractionation. Samples were taken for analysis only at the end of the exposure period.

Baker and Sellers (1957) studied electrolyte metabolism in female rats exposed to cold. Blood samples were taken at one, three, eight, sixteen, twenty-four, and forty-eight hours and at four, eight, twenty-one, and forty-five days after exposure. There was a general trend toward sodium retention in the plasma during the acclimatization period. Plasma chloride levels were generally unaltered. Potassium of the plasma was at a maximum one hour after exposure. In general, the plasma potassium dropped slowly back toward pre-exposure levels.

After forty days, the plasma volume, whole blood volume, and body water was increased to levels significantly higher than controls

of the same age. Plasma volume and whole blood volume, expressed as per cent of body weight, were still significantly greater when compared to controls of the same weight. Baker and Sellers (1957) also reported that changes in hematocrit value, hemoglobin, and plasma protein concentrations indicated a transient hemoconcentration which existed only the first few hours. Early in the period of exposure, the animals showed a loss of weight but, after eleven days, the weight increased and followed a rate somewhat less than that which they had exhibited at room temperature.

The literature dealing with the many implications of acclimatization has been voluminous, but few references to serum protein fractions under conditions of cold acclimatization have been encountered by the writer.

It seems that more data in this direction might give a better insight into liver function and general physiological adjustments that occur during the acclimatization of animals to continued cold exposure.

METHODS AND MATERIALS

1. Experimental Animal:

A. For the first part of this study the effects of short term hypothermia were investigated. A total of forty-four albino rats of both sexes was used. Twenty-three of this number served as controls. The remaining twenty-one animals were the experimental group. The weight range was from 200 to 400 grams.

B. Male and female albino rats of the Sprague-Dawley (Holtzman) strain were used in the long term experiments. One hundred thirty animals were divided into three groups, known respectively as Group A Experimental, Group B Experimental, and Group C Control. The females weighed from 230 to 260 grams and were eighteen weeks old, and weights of the males ranged from 390 to 430 grams at an age of twenty weeks at the beginning of exposure.

2. Methods of Cold Exposure and Sampling:

A. Short Term Cold Exposure

Each animal of the experimental group for the short term study was anesthetized with pentobarbital sodium (32.5 mg./kg.) and placed in a cold refrigerator (approximately $-20^{\circ}\text{C}.$) immediately after the onset of sedation. The anesthesia was helpful in reducing shivering, and therefore, less time was required to lower the animals' temperature. When the rectal temperature reached $72^{\circ}\text{F}.$ ($22.1^{\circ}\text{C}.$), usually after forty-five minutes to one hour, the animal was removed to a

refrigerator at 5°C. In this manner, the body temperature was kept constant for one hour at $21^{\circ}\text{C.} \pm 2^{\circ}\text{C.}$ Rectal temperatures were kept throughout by means of a direct reading, thermocouple type thermometer (Micron Instrument Corporation) inserted 5 centimeters into the rectum and taped firmly to the tail to prevent slippage. At the end of one hour of sustained hypothermia, blood samples (from 1 to 2 milliliters) were taken by direct heart stab. All syringes for withdrawing blood were pre-coated with mineral oil to prevent hemolysis and clotting in the syringes. The blood was allowed to clot in stoppered tubes at room temperature for two to three hours. The samples were then centrifuged at approximately 3,000 rpm. for fifteen minutes and the serum drawn off by pipetting. Serums were kept refrigerated at 5°C. until used.

B. Long Term Cold Exposure

The forty-seven animals of Group A Experimental were placed in the cold room at an initial ambient temperature of 9°C. Five days later the forty-six animals of Group B Experimental were moved into the same cold room. The temperature of the cold room had been decreased to 6°C. by small increments from the first day. The temperature had reached a final level of $4.0^{\circ}\text{C.} \pm 2.0^{\circ}\text{C.}$ by the eighth day, where it remained for the length of the experiment. A chart of the cold room temperatures is included in the section of results (Figure 1).

The rationale for dividing the cold exposed animals into two groups, five days apart, was to allow sampling at five day intervals from opposite groups. In this manner, by combining results from

Group A and Group B, values representing ten day periods of cold exposure were obtained. Therefore, samples were actually made at ten, twenty, thirty, and forty days of continued cold environmental temperature.

The animals were kept in individual cages and fed Purina Lab Chow pellets and tap water ad libitum. Fourteen animals were fed a diet of ground Purina Lab Chow with a supplement of thyroid extract (Eli Lilly and Company) which was present in the weight concentration of 0.1 per cent. This was done in an effort to increase the severity of the stress to which these animals were exposed.

Rectal temperatures were taken by random sampling at five, sixteen, twenty-three, ~~twenty-nine~~, ~~forty-one~~ and sixty-five hours, and at four, eleven, twenty-one, and forty days. The apparatus used in measuring the rectal temperatures was the same as that previously described. The thermocouples were inserted a distance of 5 centimeters in the rectum and held in place until the reading was constant (usually about four seconds).

Prior to sacrifice and removal of blood and tissues, the animals were anesthetized in the cold room with pentobarbital sodium (Nembutal). The dosages used were 32.5 mg./kg. for females and 35 mg./kg. for the males. Immediately after injection, the animals were removed to a laboratory at room temperature (25°C.) and from fifteen to thirty minutes was allowed for the anesthesia to take effect.

The surgery involved in the removal of blood and skeletal muscle consisted of an incision in the ventral wall of the animal below the

level of the liver to above the heart. The rib cage was opened, exposing the heart for withdrawal of blood directly. The skin of the left leg was removed and the overlying fascia cut away, exposing the gastrocnemius muscle. This muscle was removed, wholly or in part, and weighed on a Roller-Smith Precision balance. The weights of gastrocnemius muscles obtained in this manner ranged from 0.800 grams to 1.520 grams.

Blood was withdrawn from the heart (3 to 5 milliliters) into a syringe lightly coated with mineral oil. Blood samples were allowed to stand at room temperature for two to three hours, in tightly stoppered tubes, to insure complete clotting. They were then centrifuged for fifteen minutes at approximately 3,000 rpm. Serum was drawn off and stored in a refrigerator at 5°C. until used for electrophoretic separation, which was started that same afternoon in each instance.

The samples of gastrocnemius muscles were stored under refrigeration overnight in closed weighing bottles at 5°C. The following morning, these muscles were thoroughly homogenized in distilled water in a Ten Broeck glass tissue grinder.

The ratio of tissue weight to distilled water volume was one to one (1 gram of muscle to 1 milliliter of water). The resulting hemogenates were allowed to stand in stoppered tubes for fifteen minutes at room temperature. They were then centrifuged at approximately 3,000 rpm. for fifteen minutes. The supernatant was removed and immediately subjected to the electrophoretic examination. The method is a modification of a procedure described by Baechtel, Allen

and Dobson (1957).

3. Electrophoretic Procedure

Electrophoretic separations were carried out using a Spinco Model R Paper Electrophoresis Cell with a barbiturate buffer pH 8.6 containing diethyl barbituric acid (2.76 grams per liter) and sodium diethyl barbiturate (15.40 grams per liter). The ionic strength was 0.075. Eight strips were run simultaneously using ten lambda (0.01 milliliter) of serum sample. In the separation of the supernatant muscle extract, twenty lambda were applied to the paper strips using two applications of ten lambda each. The sample was first drawn into a micropipette from the test tube, then transferred to a Spinco sample stripper, and finally applied to the paper strip.

All serum samples were migrated for sixteen hours with a constant current of 7.5 milliamperes, giving a separation of nine to ten centimeters.

At the termination of electrophoretic separation, the strips were removed from the cell and immediately placed in a drying oven preheated to 120° to 130°C. for thirty minutes to allow complete coagulation of the protein in the strips. The drying, staining, and rinsing was according to the procedure recommended by the Spinco Division of the Beckman Instrument Company, Incorporated. (Also see Block, Durrum and Zweig, 1955, Lederer, 1955.)

The dye used on the serum samples of animals from the short term stress, and their controls, was 0.10 gram bromphenol blue and 50.0 grams zinc sulphate dissolved in fifty milliliters of 95 per cent ethanol and diluted to one liter with 5 per cent acetic acid. (Spinco

Company, Block, Durrum and Zweig, 1955.) Using this weak dye solution the length of time required for staining is six hours, and a six minute rinse in a fixative solution is required. The fixative is a solution of 3.0 grams of sodium acetate in 5 per cent acetic acid diluted to one liter. After fixing, two six minute rinsings in 5 per cent acetic acid were carried out. The strips were then blotted and removed to a drying oven preheated to 120° to 130°C. for fifteen minutes.

The eight paper strips were next removed from the drying rack and suspended in a closed container in an atmosphere saturated with ammonia fumes for six minutes. Finally, the strips were withdrawn from the ammonia and allowed to stand in the room air for at least fifteen minutes before scanning in the Spinco Analytrol (Beckman Instruments, Incorporated). The Analytrol is basically a calibrated recording photometer and automatic integrator.

The method for staining the strips of migrated proteins of the long term study, and the controls, was a slight modification and improvement over that previously described for the short term studies. This method was recently suggested by the Spinco Company and is commonly referred to as "procedure B". The dye used was 1.0 gram bromphenol blue (Eastman) diluted to one liter with absolute methanol. A pre-rinse of methanol preceded the dye bath, and staining time was thirty minutes. No fixative solution was used, but an additional six minute rinse in 5 per cent acetic acid was employed. All drying times were the same as those previously mentioned. Exposure to ammonia fumes was for fifteen minutes, after which the strips were scanned immediately.

4. Calculation of Protein Fractions

The serum protein graphs resulting from the direct photo-electric scanning were interpreted by accurately drawing lines vertically downward from the lowest points between the peaks through the saw-toothed integration record. The relative concentration of each component was determined by counting the number of integrator counts between each pair of vertical lines and comparing these individual values with the total count. (Spinco Division, Beckman Instruments, Incorporated.)

The automatically drawn graphs of the muscle extract failed to show definitive bands or peaks. Such peaks varied from strip to strip. The method for calculation described above was therefore impractical. As an alternative, the anodically moving portion of the graph was divided into five equal divisions at the zero or base line. The stationary fraction, which in every case was a definite peak, plus the small cathodically moving portion was treated as a sixth division. These divisions were determined with a Gerber Graphanalogue scale. This instrument has a calibrated coil spring scale which will divide a given length into equal or proportional segments as the scale is adjusted to coincide with the total length. The divisions were assigned arbitrary headings which were A, for the stationary and cathode component; B, for the adjacent anode segment, et cetera. Division F, therefore, is the fastest moving segment and nearest the anode side of the strip. The relative concentrations were then calculated as percentages.

5. Oxygen Consumption Studies

In order to determine whether a 0.1 per cent thyroid extract diet would affect the animals, oxygen consumption studies were made on a few animals. The apparatus used was a closed glass system connected by rubber tubing to a circuit containing a carbon dioxide absorbent (Edison Baralyme Granules). An electrically driven air pump was used to circulate the oxygen enriched air. The pressure in the system was indicated by a U-tube water manometer. As oxygen was used by the animal and as carbon dioxide was taken out of the air passing through the system, oxygen was replaced with a syringe (50 c.c.) connected to the animal container. Since the pressure in the system was constant, the volume of the replaced oxygen represented the rat's oxygen consumption. Metabolic rates were calculated in milliliters of oxygen per 100 grams per hour, corrected to standard temperature and pressure.

It should be mentioned that use of this apparatus contributes to this problem only indirectly and serves as a means of comparing metabolic rates of the normal animals with those receiving thyroid hormone.

RESULTS

In the study on short term hypothermia, the most significant serum protein pattern was a reduction in the magnitude of α_2 globulin peak. The difference was from 11.0 per cent in the controls to 8.5 per cent for the stressed animals. Beta globulins increased from control values of 19.6 per cent to 21.3 per cent ($P < .02$). Gamma globulin rose from 12.9 per cent in the control group to 15.6 per cent in the hypothermic animals ($P < .05$). Albumin and α_1 globulin were not statistically different from controls. The data for this experiment are summarized in Table I. Statistical analysis was carried out according to Edwards (1955).

The animals in the long term study showed a weight change which differed with the sex of the animals. From the graph in Figure 1, it is evident that the weights of the male rats fell with the length of exposure while the female animals showed the typical recovery and weight gain as reported by Dugal (Burton and Edholm, 1955). It is difficult to explain this as a sex difference since the male rats were two weeks older than the females. This difference will be discussed later in this manuscript.

The rectal temperatures showed an initial rise for the first sixteen hours, then a slower fall which lasted until the third day. Return to values significantly higher than controls appeared by the eleventh day.

TABLE I
SERUM PROTEIN PERCENTAGES
DURING HYPOTHERMIA

	Albumin	α_1	α_2	β	γ
<u>Controls</u> (23 animals)					
Mean	34.0	22.5	11.0	19.6	12.9
σ	± 5.40	± 4.36	± 2.60	± 1.98	± 3.42
σ_m	± 1.15	± 0.93	± 0.55	± 0.42	± 0.73
<u>Hypothermic</u> (21 animals)					
Mean	35.1	19.9	8.5	21.3	15.6
σ	± 3.82	± 4.31	± 2.28	± 2.43	± 4.30
σ_m	± 0.85	± 0.96	± 0.51	± 0.54	± 0.96
P values of difference	P > .05	P > .05	P < .01	P < .02	P < .05

These changes are also illustrated graphically in Figure 1 with the weight relationships.

The relative concentrations of the serum proteins are recorded in Tables II, III, IV, V, VI, and VII. The male and female values are averaged separately and also grouped in controls and at each ten day period. The main difference from control values is the decreased albumin fraction in the experimental groups. From Table Va and b, it can be seen that this decrease is greatest in the male animals and that the minimum level in both sexes occurs near the thirtieth day of exposure.

A significant increase in α_2 and decrease in gamma-globulin occurs in the females at the ten day period (Table IIIa and b). The males show no significant changes at this time.

During the twenty day period, male rats showed a decrease in the albumin fraction and an increase in the antibody-carrying protein, gamma globulin. α_2 globulin remains significantly increased at twenty days in the females, but the levels of serum albumin are not statistically different (Table IVa and b).

At the thirty day period (Table Va and b), the picture is somewhat confusing. With the extreme decrease in the albumin peak, the other relative values show an increase which may be due to the albumin decline. Therefore, rather than indicating actual increases in the globulins, the data support the conclusion that the decrease in albumin is a marked one. Inspection of the serum protein curves clearly indicates this (Figure 2). The percentage of gamma globulin is not significantly different from the values of control animals.

TABLE IIa
SERUM PROTEIN PERCENTAGES
CONTROLS

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Females</u>					
C-2	28.1	14.9	12.1	24.5	20.4
C-3	29.6	19.4	16.2	15.3	19.4
C-4	31.0	16.3	15.2	21.4	16.1
C-7	32.7	15.2	16.5	19.1	16.5
C-13	25.3	13.4	9.7	21.8	29.8
C-14	34.7	15.4	8.4	18.0	23.4
C-17	28.1	17.5	10.3	19.2	24.9
C-20	29.7	18.6	16.2	17.4	18.0
C-24	32.7	14.0	14.3	23.3	14.7
C-25	35.7	14.2	10.2	19.7	20.2
C-26	33.1	15.4	13.3	19.2	19.0
C-27	27.6	12.2	11.0	29.5	19.7
C-28	31.8	15.7	11.0	24.9	16.6
C-29	37.4	10.9	12.3	19.9	19.5
C-43	28.9	24.7	9.4	23.4	13.6
C-44	34.8	21.1	11.3	16.2	16.7
C-45	39.2	18.9	13.2	18.9	9.9
Mean	31.8	16.3	12.4	20.7	18.7
σ	± 3.66	± 3.31	± 2.49	± 3.52	± 4.41
σ_m	± 0.92	± 0.83	± 0.62	± 0.88	± 1.10

TABLE IIb
SERUM PROTEIN PERCENTAGES
CONTROLS

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Males</u>					
C-15	24.7	17.4	10.5	24.7	22.7
C-16	27.2	17.3	13.3	22.0	20.2
C-18	27.4	20.9	13.0	21.1	17.6
C-19	32.0	17.6	13.2	22.0	15.3
C-21	28.1	19.5	12.7	17.3	22.4
C-22	32.8	18.5	13.5	16.7	18.1
C-30	33.2	17.6	14.4	19.5	15.3
C-31	29.1	18.5	17.4	17.7	17.4
C-32	33.3	18.4	15.2	20.0	13.0
C-33	28.0	16.8	18.8	19.1	17.4
C-34	32.0	17.3	18.5	21.4	10.9
C-35	31.7	20.1	17.1	19.5	11.6
C-36	30.4	21.2	13.7	19.6	15.0
C-37	33.0	18.2	16.4	17.3	15.1

(continued next page)

TABLE IIb (continued)

Animal Numbers	Albumin	α_1	α_2	β	γ
C-40	30.0	24.6	12.1	21.4	11.7
C-41	35.9	23.6	7.5	17.2	14.0
C-42	26.0	19.4	14.3	27.6	12.7
Mean	30.3	19.2	14.2	20.2	15.9
σ	± 2.97	± 3.26	± 2.80	± 2.79	± 3.47
σ_m	± 0.74	± 0.82	± 0.70	± 0.70	± 0.87
P values for difference between males and females	P > .05	P > .05	P > .05	P > .05	P > .05
Data of males and females grouped	Mean 31.0	17.8	13.3	20.5	17.3
	σ ± 3.42	± 3.15	± 2.80	± 2.07	± 4.21
	σ_m ± 0.60	± 0.55	± 0.49	± 0.36	± 0.73

TABLE IIIa
SERUM PROTEIN PERCENTAGES
AFTER 10 DAYS OF COLD EXPOSURE

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Females</u>					
23	41.1	10.5	13.5	18.1	16.7
28	36.0	13.2	16.9	21.1	12.8
32	32.7	18.6	18.3	16.3	14.1
82	30.0	15.0	16.3	25.0	13.7
89	36.9	15.0	14.1	19.3	14.7
75	31.4	12.1	22.1	18.6	15.9
Mean	34.7	14.1	16.9	19.7	14.6
σ	± 3.75	± 2.57	± 2.85	± 2.75	± 1.32
σ_m	± 1.68	± 1.15	± 1.27	± 1.25	± 0.59
P values of difference from controls	$P > .05$	$P > .05$	$P < .01$	$P > .05$	$P < .01$

TABLE IIIb
SERUM PROTEIN PERCENTAGES
AFTER 10 DAYS OF COLD EXPOSURE

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Males</u>					
12	31.7	16.2	19.8	21.6	10.8
8	34.4	19.1	17.6	17.6	11.4
4	32.5	14.5	14.1	27.0	11.4
53	21.4	18.1	20.1	23.0	17.5
64	20.9	22.4	18.2	22.1	16.4
70	34.9	16.1	11.1	20.8	17.1
Mean	29.3	17.7	16.8	22.0	14.1
σ	± 5.86	± 2.56	± 3.22	± 2.80	± 2.92
σ_m	± 2.62	± 1.14	± 1.44	± 1.25	± 1.31
P values of difference from controls	P > .05	P > .05	P > .05	P > .05	P > .05
P values for difference between males and females	P > .05	P > .05	P > .05	P > .05	P > .05

(continued next page)

TABLE IIIb (continued)

TABLE IIIb (continued)						
Albumin		α_1	α_2	β	γ	
Data of males and females grouped	Mean	32.0	15.9	16.8	20.9	14.4
	σ	± 5.59	± 3.06	± 3.04	± 3.00	± 2.28
	σ_m	± 1.69	± 0.92	± 0.92	± 0.90	± 0.69
P values of difference from controls		P>.05	P>.05	P<.01	P>.05	P<.01

TABLE IVa

SERUM PROTEIN PERCENTAGES
AFTER 20 DAYS OF COLD EXPOSURE

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Females</u>					
29	30.0	15.5	17.5	19.9	17.2
34	26.6	12.7	17.9	26.6	16.2
37	27.0	14.4	16.5	27.3	14.7
42	24.6	15.6	20.1	24.1	15.6
27	32.9	13.2	12.7	27.2	14.0
76	32.9	15.3	16.5	17.3	18.0
77	30.2	12.7	17.8	19.3	20.0
92	22.1	15.7	15.7	30.1	16.5
93	31.3	20.9	9.3	21.3	17.2
Mean	28.6	15.1	16.0	23.7	16.6
σ	± 3.55	± 2.36	± 3.02	± 4.15	± 1.69
σ_m	± 1.26	± 0.83	± 1.07	± 1.47	± 0.60
P values of difference from controls	P > .05	P > .05	P < .01	P > .05	P > .05

TABLE IVb
SERUM PROTEIN PERCENTAGES
AFTER 20 DAYS OF COLD EXPOSURE

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Males</u>					
9	26.2	20.8	15.0	19.6	18.3
16	21.0	15.0	21.7	24.3	18.0
60	22.1	22.1	13.4	22.5	19.9
55	29.6	16.8	12.8	21.4	19.4
65	25.0	14.2	17.1	22.8	20.9
Mean	24.8	17.8	16.0	22.1	19.3
σ	± 3.06	± 3.14	± 3.22	± 1.56	± 1.06
σ_m	± 1.53	± 1.57	± 1.61	± 0.78	± 0.53
P values of difference from controls	$P < .01$	$P > .05$	$P > .05$	$P > .05$	$P < .01$
P values for difference between males and females	$P > .05$	$P > .05$	$P > .05$	$P > .05$	$P < .01$

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TABLE IVb (continued)

		Albumin	α_1	α_2	β	γ
Data of males and females grouped	Mean	27.2	16.1	16.0	28.1	17.6
	σ	± 3.85	± 2.95	± 3.09	± 3.54	± 1.98
	σ_m	± 1.03	± 0.82	± 0.86	± 0.98	± 0.55
P values of difference from controls		$P < .01$	$P > .05$	$P < .01$	$P > .01$ $< .05$	$P > .05$

TABLE Va
SERUM PROTEIN PERCENTAGES
AFTER 30 DAYS OF COLD EXPOSURE

Animal Numbers	Albumin	α_1	α_2	β	γ
Females					
38	31.3	15.5	14.6	25.6	12.9
26	15.1	28.6	19.4	16.3	20.6
35	27.4	14.1	22.5	22.8	13.3
95	20.6	28.9	14.0	25.9	10.5
79	17.1	20.6	17.7	28.8	15.7
Mean	22.3	21.5	17.6	23.9	14.6
σ	± 6.14	± 6.27	± 3.14	± 4.24	± 3.42
σ_m	± 3.07	± 3.17	± 1.57	± 2.12	± 1.71
P values of difference from controls	P < .01	P > .05	P < .01	P > .05	P > .05

TABLE Vb
SERUM PROTEIN PERCENTAGES
AFTER 30 DAYS OF COLD EXPOSURE

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Males</u>					
15	11.8	22.0	26.5	26.0	13.7
3	13.1	21.0	20.6	25.4	19.9
14	23.9	22.2	13.0	22.2	18.7
13	16.3	24.3	23.4	23.7	12.4
50	20.9	25.3	21.5	18.9	13.4
57	7.3	24.0	19.9	28.8	19.9
52	16.1	18.3	23.4	24.3	17.8
72	12.4	20.6	25.4	20.6	10.9
Mean	15.2	22.2	21.7	25.0	15.8
σ	± 4.96	± 2.14	± 3.92	± 3.43	± 3.39
σ_m	± 1.87	± 0.81	± 1.48	± 1.30	± 1.28
P values of difference from controls	P < .01	P < .02	P < .01	P < .01	P > .05
P values for difference between males and females	P > .05	P > .05	P > .05	P > .05	P > .05

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TABLE Vb (continued)

		Albumin	α_1	α_2	β	γ
Data of males and females grouped	Mean	17.9	22.0	20.1	24.6	15.4
	σ	± 6.44	± 4.25	± 4.14	± 3.80	± 3.45
	σ_m	± 1.86	± 1.23	± 1.20	± 1.10	± 1.00
P values of difference from controls		$P < .01$	$P < .01$	$P < .01$	$P < .01$	$P > .05$

This thirty day period exhibited the most dramatic changes in the serum protein picture.

The results from the terminal or forty day studies indicate that the electrophoretic pattern is returning to normal in the females, although it would appear that gamma globulin levels are low. The albumin percentages are not significantly lower than controls; the antibody or gamma fraction is significantly lower. The males do not show a return to normal levels of serum albumin. Furthermore, their gamma component is the same as the controls. The increases seen in the other fractions, since they are percentage values, may be relative increases only. (Table VIa and b). The changes in the serum protein patterns are summarized graphically in Figure 3.

From Table VII, it is clear that the animals maintained on a 0.1 per cent thyroid extract diet in the cold showed no different responses of serum protein patterns than animals given the normal diet. Oxygen consumption studies on these two groups revealed no significant differences.

The fluctuations in the arbitrary divisions of muscle extract at the four sampling periods failed to show any marked characteristics. These values appear in Tables VIII through XIII.

The relative area of peaks E and F, the fastest moving components, showed a significant increase on the tenth day. Peak B was significantly decreased. No other changes were significant (Table IX).

At the twenty day period, all components showed no statistical difference from control values (Table X).

TABLE VIa
SERUM PROTEIN PERCENTAGES
AFTER 40 DAYS OF COLD EXPOSURE

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Females</u>					
24	28.8	18.0	18.3	22.5	12.4
36	31.7	15.2	21.4	18.6	13.1
41	29.5	16.6	24.5	21.6	7.9
45	29.8	18.7	14.4	25.1	12.0
40	18.6	21.0	18.9	27.8	13.7
47	34.9	12.1	16.9	22.3	13.8
94	24.0	14.6	21.0	26.1	14.3
Mean	28.2	16.6	19.3	23.4	12.4
σ	± 4.95	± 2.72	± 3.05	± 2.87	± 2.00
σ_m	± 2.02	± 1.11	± 1.25	± 1.17	± 0.82
P values of difference from controls	P > .05	P > .05	P < .01	P > .05	P < .01

TABLE VIb
SERUM PROTEIN PERCENTAGES
AFTER 40 DAYS OF COLD EXPOSURE

Animal Numbers	Albumin	α_1	α_2	β	γ
<u>Males</u>					
17	9.2	21.2	26.9	26.1	16.6
62	21.3	25.6	12.9	25.7	14.9
68	23.1	29.5	12.8	20.8	13.8
51	14.8	21.2	22.7	27.0	14.2
69	19.8	18.4	20.8	27.3	13.7
66	16.0	20.3	19.9	24.9	18.9
63	19.1	21.5	18.4	24.2	16.7
Mean	17.6	22.5	19.2	25.1	15.5
σ	± 4.34	± 3.48	± 4.72	± 2.04	± 1.79
σ_m	± 1.77	± 1.42	± 1.93	± 0.83	± 0.73
P values of difference from controls	P < .01	P > .05	P < .05	P < .01	P > .05
P values for difference between males and females	P < .01	P < .01	P > .05	P > .05	P < .02

(continued next page)

TABLE VIb (continued)

		Albumin	α_1	α_2	β	γ
Data of males and females grouped	Mean	22.9	19.6	19.3	24.3	14.0
	σ	± 7.04	± 4.31	± 3.98	± 2.64	± 2.45
	σ_m	± 1.95	± 1.20	± 1.10	± 0.73	± 0.68
P values of difference from controls		$P < .01$	$P > .05$	$P < .01$	$P < .01$	$P < .01$

Relative Changes of Serum Protein Fractions
During Acclimatization to Cold

FIGURE 3

TABLE VII

SERUM PROTEIN PERCENTAGES
AFTER 42-45 DAYS OF COLD EXPOSURE
.1% THYROID EXTRACT IN DIET

Animal Numbers		Albumin	α_1	α_2	β	γ
Male	19	20.4	20.1	20.1	21.7	17.6
Female	30	23.1	18.8	15.6	29.7	12.8
Female	25	22.8	17.8	17.8	26.9	14.7
Male	22	24.6	15.7	17.5	23.7	18.6
Female	90	19.7	17.7	18.5	27.1	17.1
Male	85	24.6	17.1	20.7	25.8	11.8
Female	78	27.4	14.5	17.5	25.8	14.8
Male	86	26.3	15.6	19.2	24.6	14.4
Female	96	20.8	16.7	21.8	25.0	15.7
Mean		23.3	17.1	18.7	25.6	15.3
σ		± 2.52	± 1.63	± 1.80	± 2.13	± 2.09
σ_m		± 0.89	± 0.58	± 0.64	± 0.75	± 0.74
P values of difference from normal diet at 40 days		$P > .05$	$P > .05$	$P > .05$	$P > .05$	$P > .05$

TABLE VIII

MUSCLE PROTEIN PERCENTAGES
CONTROLS

Animal Numbers		Division:	A	B	C	D	E	F
Female	C-1		31.9	23.2	18.8	15.9	8.7	1.5
Female	C-2		29.2	20.8	14.9	16.2	14.9	3.9
Female	C-3		21.1	22.6	21.1	15.8	12.8	6.8
Female	C-4		19.6	23.2	22.3	17.9	12.5	4.5
Female	C-5		24.0	19.0	19.8	18.2	13.2	5.8
Female	C-6		43.1	18.2	13.1	10.9	9.5	5.1
Female	C-7		28.8	22.1	19.2	17.3	9.6	2.9
Female	C-8		32.4	23.8	18.1	13.3	9.5	2.9
Male	C-30		23.6	26.4	21.7	17.0	7.1	4.2
Male	C-31		21.1	23.8	23.4	15.2	12.5	4.0
Male	C-32		20.2	25.5	22.3	15.4	12.1	4.5
Male	C-33		19.5	25.4	23.4	16.8	10.9	3.9
Male	C-34		22.1	25.4	23.0	15.0	10.3	4.1
Male	C-35		19.7	24.3	21.6	17.0	13.3	4.1
Male	C-36		24.8	22.9	18.7	15.4	13.1	5.1
Male	C-37		22.9	22.4	20.2	16.6	13.0	4.9
		Mean	25.2	23.1	20.1	15.8	11.4	4.3
		σ	± 6.21	± 2.20	± 2.85	± 1.74	± 2.05	± 1.18
		σ_m	± 1.60	± 0.57	± 0.74	± 0.45	± 0.53	± 0.30

TABLE IX

MUSCLE PROTEIN PERCENTAGES
AFTER 10 DAYS OF COLD EXPOSURE

Animal Numbers		Division: A	B	C	D	E	F
Male	12	25.7	18.7	19.3	15.0	13.9	7.5
Male	53	14.4	22.4	14.8	16.8	13.9	7.7
Female	28	21.9	17.9	16.8	18.9	15.8	8.7
Female	39	18.9	18.9	18.9	19.4	16.6	7.4
Male	32	26.2	15.1	17.8	16.9	16.0	8.0
Female	23	23.2	15.5	19.3	17.7	15.5	8.8
Male	8	27.0	18.1	16.2	16.2	16.2	6.4
Male	11	29.1	13.7	15.1	16.1	16.8	9.1
Male	4	24.3	18.7	17.3	15.9	16.4	7.5
Mean		23.4	17.7	18.4	17.0	15.7	7.9
σ		± 4.26	± 2.43	± 2.64	± 1.36	± 1.01	± 0.80
σ_m		± 1.51	± 0.86	± 0.93	± 0.48	± 0.36	± 0.28
P values of difference from controls		P > .05	P < .01	P > .05	P > .05	P < .01	P < .01

TABLE X
MUSCLE PROTEIN PERCENTAGES
AFTER 20 DAYS OF COLD EXPOSURE

Animal Numbers		Division: A	B	C	D	E	F
Female	93	23.2	21.0	22.1	19.3	9.9	4.4
Male	65	20.6	23.2	21.1	20.1	12.9	2.1
Male	73	22.7	20.2	19.3	20.2	12.9	4.7
Female	92	21.5	29.7	18.7	15.9	10.6	3.7
Female	76	20.5	25.5	19.1	18.6	11.4	5.0
Male	60	27.8	21.2	17.0	20.1	10.8	3.1
Female	77	24.2	28.8	19.2	11.6	11.1	5.1
Male	55	21.0	24.1	19.0	18.5	14.9	2.6
Female	37	22.6	22.6	19.2	17.8	13.9	3.8
Female	42	25.9	27.3	20.8	14.4	10.2	1.4
Male	10	27.9	24.5	17.3	14.9	11.5	3.8
Female	27	22.5	23.4	23.4	17.7	11.0	1.9
Female	34	30.1	19.3	17.4	14.7	13.9	4.6
Male	16	24.8	19.9	21.2	14.2	14.2	5.8
Male	9	18.4	23.8	21.1	18.0	13.7	5.1
Female	29	26.9	26.6	18.8	15.0	10.9	1.9
Mean		23.8	23.8	19.7	16.9	12.1	3.7
σ		± 3.12	± 3.04	± 1.74	± 2.50	± 1.57	± 1.33
σ_m		± 0.81	± 0.78	± 0.45	± 0.65	± 0.41	± 0.34
P values of difference from controls		P > .05	P > .05	P > .05	P > .05	P > .05	P > .05

Perhaps the greatest change in the electrophoretic pattern of the muscle protein extract appeared at the thirty day period. An increase in the stationary component (peak A) from a control percentage of 25.2 to the experimental value of 32.6 per cent is significant. Division C showed a decrease which is significant. Division F, the fastest moving component, is significantly lower than control levels. These values are in Table XI. Figure 4 shows the comparison between muscle protein patterns of controls and at thirty days of cold exposure. Samples which are typical of each group are presented.

The relative concentrations of the protein fractions of muscle extract at the terminal or forty day period differ from controls only in an increase in component B and a decrease in component E (Table XII).

The muscle fractions of the thyroid treated animals show no difference from the animals maintained on the normal diet. These data are presented in Table XIII. Graphic illustration of muscle fraction changes is presented in Figure 5.

Some gross or qualitative observations deserve mention here that may be important to the work presented. The general health of all the animals, at the end of the exposure, was poor. Ears and tails were necrotic, and had been since about the thirtieth day. In many instances, portions of the animals' tails had been chewed away. Edema was noted in the rats' tails from about the twenty-fifth to the thirtieth days. The incidence of edema was nearly equal for both sexes. The number of animals with chewed tails was much greater in the male group. The only females which illustrated this were among the thyroid treated group.

TABLE XI
MUSCLE PROTEIN PERCENTAGES
AFTER 30 DAYS OF COLD EXPOSURE

Animal Numbers		Division: A	B	C	D	E	F
Male	13	30.2	18.7	16.0	19.1	13.8	2.2
Female	26	25.7	24.3	17.5	20.4	10.2	1.9
Female	35	33.9	20.3	13.6	16.1	12.8	3.3
Male	3	26.1	23.9	19.9	16.3	11.6	2.2
Male	14	24.8	24.4	16.5	16.9	12.4	5.0
Female	44	33.0	22.3	16.2	14.2	10.2	4.1
Male	15	38.5	18.7	13.4	16.4	9.5	3.4
Female	38	17.6	26.2	20.5	20.1	13.5	2.0
Male	58	36.0	23.4	15.7	13.7	8.6	2.5
Female	95	34.3	22.9	18.8	15.1	9.1	0.7
Female	79	34.7	21.3	20.3	16.8	6.2	0.7
Male	57	31.3	27.4	13.9	13.5	11.7	2.2
Male	50	34.2	26.5	15.8	15.0	7.3	1.3
Male	52	45.5	20.6	12.2	13.8	6.9	1.1
Female	81	39.3	22.7	14.2	15.6	7.6	0.5
Male	72	36.4	21.5	14.6	15.8	10.5	1.2
Mean		32.6	22.8	16.2	16.2	10.1	2.1
σ		± 6.46	± 2.52	± 2.50	± 2.07	± 2.36	± 1.24
σ_m		± 1.67	± 0.65	± 0.65	± 0.53	± 0.61	± 0.32
P values of difference from controls		P < .01	P > .05	P < .01	P > .05	P > .05	P < .01

Muscle Protein Patterns
Of Control and After
30 Days of Cold Exposure

FIGURE 4

TABLE XII

MUSCLE PROTEIN PERCENTAGES
AFTER 40 DAYS OF COLD EXPOSURE

Animal Numbers		Division: A	B	C	D	E	F
Female	47	23.0	21.5	19.3	15.3	14.6	6.2
Female	25	22.5	19.5	20.1	17.8	14.2	5.9
Female	40	22.2	21.8	20.8	17.1	15.3	2.8
Female	45	27.3	19.5	18.8	16.8	13.7	3.9
Male	17	24.9	24.5	18.7	16.6	12.9	2.5
Female	41	24.3	17.6	19.5	17.6	14.8	6.2
Female	36	30.2	16.5	19.8	18.7	13.2	1.6
Female	24	37.1	17.4	15.0	14.6	13.1	2.8
Mean		26.4	19.8	19.0	16.8	14.0	4.0
σ		± 4.75	± 2.52	± 1.64	± 1.25	± 0.83	± 1.74
σ_m		± 1.80	± 0.95	± 0.62	± 0.47	± 0.31	± 0.66
P values of difference from controls		P > .05	P < .01	P > .05	P > .05	P < .01	P > .05

TABLE XIII

MUSCLE PROTEIN PERCENTAGES
AFTER 42-45 DAYS OF COLD EXPOSURE
.1% THYROID EXTRACT IN DIET

Animal Numbers		Division: A	B	C	D	E	F
Female	78	29.9	20.8	18.6	14.9	12.2	3.6
Female	43	24.9	22.5	16.7	15.8	16.3	3.8
Female	25	35.7	17.9	14.5	15.5	11.6	4.8
Male	19	33.5	16.0	15.1	16.5	14.2	4.7
Female	30	31.2	20.4	21.6	16.7	9.0	1.2
Male	22	31.8	21.8	17.9	14.5	10.1	3.9
Female	90	28.7	20.0	18.7	16.7	12.0	4.0
Male	85	26.8	18.2	16.7	17.2	15.3	5.7
Mean		30.3	19.7	17.5	16.0	12.6	4.0
σ		± 3.29	± 2.04	± 2.11	± 0.90	± 2.35	± 1.23
σ_m		± 1.24	± 0.77	± 0.80	± 0.34	± 0.89	± 0.46
P values of difference from normal diet at 40 days		P > .05	P > .05	P > .05	P > .05	P > .05	P > .05

Relative Changes of Muscle Protein Fractions
During Acclimatization to Cold

FIGURE 5

DISCUSSION

The significant decrease in serum α_2 globulin ($P < .01$) from a control value of 11.0 per cent to the hypothermic level of 8.5 per cent in animals subjected to short term cold exposure indicates an imbalance in the dynamic state of this serum protein. It is unlikely that urinary excretion of this fraction could account for this decrease, since the smaller albumin molecule probably would be lost also. Indeed, any passage of the α_2 fraction from the blood through the kidney is improbable in the face of albumin retention. It would seem that α_2 synthesis in the liver is retarded. Since all the proteins in plasma owe their presence to the activity of liver or reticuloendothelial tissue (White, Handler, Smith and Stetten, 1954), and cellular activity is generally retarded in the hypothermic animal, perhaps the particular liver cells responsible for α_2 globulin production are most susceptible to inhibition by decreased temperature. The exact mechanism for such inhibition is not clear. Increases in beta ($P < .02$) and gamma globulin ($P < .05$) indicate increased synthesis of these fractions by the liver. This would suggest that the liver is still metabolizing during the hypothermic state.

Long term exposure of rats to cold resulted in a marked alteration in the electrophoretic pattern of serum proteins. The most dramatic change was the decreased percentages of serum albumin throughout the acclimatization process. Present knowledge of the role of albumin

in the normal animal is limited. The main function of this protein fraction is believed to be that of osmotic pressure regulation. Because it is the smallest molecule, it contributes 75 to 80 per cent of the total osmotic effect of the plasma proteins (White, Handler, Smith and Stetten, 1954). A decrease in serum albumin is associated with many kinds of disease, especially those accompanied by wasting or malnutrition or an acute febrile illness (Walker, Boyd and Asimov, 1954). Under the same circumstances there is usually some elevation of the beta globulins and alpha globulins. These changes occur in so many conditions that they are seldom useful for diagnostic purposes, but they are of value in prognosis since the changes over a period of time can be used for estimating the severity of the condition or the trend of the disorder (White, Handler, Smith and Stetten, 1954).

The animals in the cold were probably maintaining a metabolism very close to maximum capacity. This supposition is supported by the fact that normal and thyroid treated animals showed no difference in rates of oxygen consumption. It seems reasonable to assume that animals provided with this level of thyroid hormone in the diet responded by decreasing the output of that hormone from the thyroid gland and thereby remained at the metabolic levels which were necessary to meet the stimulus of a cold environment.

One may speculate on the decrease of serum albumin reported here. Such a decrease could result from a reduced dietary intake of protein, increased urinary excretion of albumin, a greater catabolism of albumin to furnish tissue proteins or energy, or finally a decreased synthesis of albumin by the liver. Since dietary intake has been

reported as increasing 60 per cent during cold exposure (Burton and Edholm, 1955) this causative factor may be discounted with the exception that this does not eliminate the possibility of decreased absorption from the gut. Excessive urinary loss of albumin during acclimatization to cold has not been indicated in the literature. Although it may occur to some extent in early stages of cold exposure (Burton and Edholm, 1955), it seems improbable that it could account for the decrease seen in this experiment. The work of Wasserman and Mayerson (1951) showed no selective tissue uptake of dog serum albumin at room temperature, labeled with radioactive iodine, but breakdown products of albumin were found in bile and urine in quantities which indicate these as the main avenues of elimination. The tissues studied by Wasserman and Mayerson were bone, brain, fat, pancreas, bone marrow, diaphragm, skeletal muscle, spleen, gall bladder, intestine, stomach, lymph node, heart, liver, mesentary, skin, kidney, bladder, and lung. Furthermore, similar experiments by Campbell and Stone (1957) and Humphrey, Neuberger and Perkins (1957) suggest that serum albumin is relatively inactive as a precursor of tissue proteins and that there is no indication of any preferential utilization of albumin by one tissue over another. It should be pointed out, however, that the relationships were studied only in normal animals at room temperature.

From the indications of the above work, the role of serum albumin for synthesis of tissue proteins is an improbable explanation of its observed decrease in the cold exposed animals of the present study. Furthermore, Heroux (1958) has reported that muscle protein also declines in animals exposed four weeks to cold. The alternative

conclusion then, is that the albumin levels of serum have decreased due to a greater utilization of proteins as energy supplies to maintain the higher metabolic activity which is necessary for animals in a cold environment. It remains to be shown that actual liver synthesis of albumin is less or whether existing serum albumin is catabolized to furnish the extra energy source. Since the albumin levels of females increased from 22.3 per cent at thirty days to 28.2 per cent at forty days in the cold room, it would appear that the liver still has the capacity of synthesis. It is possible that liver production of serum albumin lags behind its increased catabolism, resulting in lower existing serum levels. The terminal rise may be an indication that liver synthesis has been accelerated or that utilization has decreased to restore normal levels of the protein fraction for its role of osmotic pressure regulation in the blood. The albumin may be an appropriate index and reflect liver function during the acclimatization process.

The more pronounced albumin decrease in the male rats than in females throughout cold exposure was statistically significant only at the forty day time interval. Male serum albumin concentration was 17.6 per cent and female serum albumin was 28.2 per cent of the total protein (Tables VIa and b). These data suggest that the male animals were not yet acclimatized. This correlates with the failure of the male animals to maintain their body weights during cold exposure. Before further discussion of the serum protein relationships is undertaken, mention should be made of the difference between the males and females as regards the weight responses during cold acclimatization.

The female animals showed a loss in weight and then a return to normal weight gain which agrees with reports in the literature previously cited (Burton and Edholm, 1955). The male rats showed a decline of weight at the ten day period followed by an increase at the twentieth day which did not reach control levels. Progressive weight losses were observed at the thirty and forty day time intervals (Figure 1). This would indicate that two populations were under study. Apparently, the male animals were unable to meet the demands of cold stress and after passing through the initial period of resistance, failed to achieve the state of recovery. The factor responsible for this difference may have been the advanced age of the male rats (i.e. two weeks older than females) and that they had reached nearly full growth at the beginning of cold exposure. The suggestion that older animals are unable to acclimatize has been made by Weiss (1954), who used in vitro tissue metabolism as the criterion for acclimatization. Whether the difference in the present study is due to sex or to age cannot be stated from the data.

Higher levels of alpha and beta globulins relative to the decreased albumin levels during the various periods of acclimatization may be indicative of an increased activity of the particular liver cells or enzyme systems that are responsible for the synthesis of these fractions. The increase in liver metabolism of cold exposed animals reported by several authors (You and Sellers, 1951, Weiss, 1954, Clark, Chinn, Ellis, Powel and Criscuolo, 1954, Weiss, 1957, Hannon, 1958) may be responsible for the increase of these globulins observed in the present study.

The gamma globulin fraction in the females at ten days (14.6 per cent) and at forty days (12.4 per cent), was lower than controls (18.7 per cent). The difference was significant in both cases ($P < .01$). This antibody carrying fraction, however, was elevated in the males from control values of 15.9 per cent to 19.3 per cent after twenty days of cold exposure. Since antibodies are mainly associated with the gamma globulins, a rise in this fraction generally accompanies a bacterial infection (White, Handler, Smith and Stetten, 1954). This is interesting in the light of the higher gamma fraction of the male animals at twenty days. Although the presence of an infection is not specifically concluded from these data, the elevated gamma fraction in the male rat suggests that possibility.

The reason for lower percentage of gamma globulins in the female at ten and forty days is not clear. This fraction owes its existence to the reticuloendothelial system (spleen, liver, lymph nodes, bone marrow, et cetera). The findings of Heroux and Gridgeman (1958) which show a reduction in growth of the spleen during continued cold exposure may be of significance concerning the gamma decrease of the younger, growing females.

Very little work appears in the literature on the paper electrophoretic behavior of water extractable proteins of muscle. Jacob (1947) and Helander (1957) have studied them by the moving boundary method in a liquid medium. Definite peaks which represent the sarcoplasmic proteins were observed by this method. The sarcoplasmic proteins are myogen, myoalbumin, globulin x, and myoglobin. Only the sarcoplasmic proteins are water soluble.

Baechtel, Allen and Dobson (1957) have reported differences in paper electrophoretic behavior of water soluble muscle proteins due to vitamin E deficiency, but made no mention of specific protein components other than their relative speed of mobility across the paper strip.

A search of the literature has revealed no statement of specific mobilities, using paper electrophoresis, in terms of the sarcoplasmic proteins. Apparently this area of endeavor has not been thoroughly investigated. According to Helander (1957), it may be that paper electrophoretic mobilities differ from the moving boundary method in a liquid medium.

Data collected in this experiment did show a change in the electrophoretic pattern of muscle proteins of rats in different stages of cold acclimatization. The most salient effect was seen at the thirty day period (Figure 4). The stationary fraction increased significantly to 32.6 per cent from a control value of 25.2 per cent, while the fastest moving fraction showed a significant decrease. This fraction was 4.3 per cent in controls and 2.1 per cent after thirty days. It is generally agreed that smaller protein molecules move fastest and the larger molecules move slowest, if the isoelectric points of both types of molecules are the same. Adsorption on the paper at the point of application would modify this relationship, however. Furthermore, the isoelectric points of muscle proteins vary from pH 3.5 to 7.0 (Helander, 1957). It seems wise, therefore, to be cautious in making speculations as regards specific proteins in discussing the relationships of the electrophoretic data in the present study. A correlation seems apparent, however, in that the most

pronounced alteration of the muscle protein fractions occurred at the same time period which exhibited the greatest alteration in the serum protein fractions. It would appear that the study of imbalance in protein metabolism would prove fruitful in future research on animals exposed to continued cold stress.

The increase in colonic temperatures at sixteen hours ($P < .01$) indicates that heat production was great enough to cause a slight rise during the initial cold exposure. Energy stores in the form of carbohydrates and fats were available at the beginning of cold exposure. The downward trend of the rectal temperatures until sixty-five hours of exposure suggests reduced ability for heat retention. After eleven days, however, significantly higher body temperatures were encountered ($P < .01$). Fregley (1953) has reported statistically higher colonic temperatures after ten days of continuous cold exposure. It appears that the animals in the present study had regained sufficient heat retention and from the previously mentioned alterations in serum albumin levels, utilization of proteins for energy production is suggested. This hypothesis is supported by the findings of Heroux (1958) and Heroux and Gridgeman (1958) that muscle proteins are decreased after four weeks and eight weeks of exposure to 6°C .

The colonic temperatures in the present study had fallen to control levels after forty days of cold exposure, indicating that acclimatization was achieved regarding body temperature. It is evident, however, that adjustments made by different tissues or systems vary considerably. Therefore, in any statement or definition of acclimatization to cold, one should consider the individual criteria for

acclimatization. Complete understanding of cold acclimatization will depend on further knowledge of all functional parameters, and no single function can be used as a measure of acclimatization to a cold environment.

SUMMARY

1. Relative concentrations of the serum proteins of rats during short term hypothermia (two hours) exhibited changes in α_2 , beta, and gamma globulins. α_2 globulin concentrations decreased 23 per cent during hypothermia ($P < .01$). The beta fraction rose 11 per cent ($P < .02$) and gamma was elevated 21 per cent ($P < .05$) during hypothermia.

2. Continued exposure to cold for forty days resulted in a marked decrease of serum albumin. After thirty days, serum albumin concentration of male rats was 50 per cent lower ($P < .01$), while the decrease in the females was only 30 per cent lower than controls ($P < .01$). Samples taken after forty days of acclimatization showed that albumin in the males was still significantly less than control values ($P < .01$) but the albumin of females had risen back to control levels. Increased relative concentrations of serum alpha and beta globulins were observed at different time periods. Male rats exhibited a 21 per cent increase in gamma globulin at twenty days. Female rats had decreased gamma fractions after ten days and forty days.

3. Electrophoretic patterns of the water soluble extracts of rat muscle showed the greatest alterations after thirty days exposure. The stationary protein component increased 30 per cent over control values. The fastest moving component was decreased by 50 per cent.

4. The serum protein patterns of rats maintained on a thyroid supplemented diet did not vary from those receiving a normal diet.

5. The electrophoretic patterns of the muscle proteins of thyroid treated animals failed to show statistical difference from non-treated animals.

6. Changes in rectal temperatures and body weights during forty days of cold exposure agree with findings cited in the literature.

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