THE UNIVERSITY OF MANITOBA

THE EFFECT OF COLD EXPOSURE AND COLD ACCLIMATIZATION ON PROTEIN AND DNA SYNTHESIS IN A RODENT (MICROTUS PENNSYLVANICUS).

BY

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
IUDR	Iododeoxyuridine
dTTP	Thymidine triphosphate
dTDP	Thymidine diphosphate
dTMP	Thymidine monophosphate
PCA	Perchloric acid
TCA	Trichloracetic acid
KOH	Potassium hydroxide

ABSTRACT

The purpose of this study was to investigate the effects of cold exposure and seasonal cold acclimatization on the incorporation of ^{3}H-thymidine and ^{14}C-amino acids into deoxyribonucleic acid and protein, respectively, of various tissues in the meadow vole (Microtus pennsylvanicus). Voles were captured in the field in July and December, held at 20°C overnight and exposed to either 15°C or 27°C immediately after intraperitoneal injection of the above radioactive precursors. Incorporation of the precursors into DNA and protein was inferred from changes in specific radioactivity up to 6 hours after intraperitoneal injection.

In summer, the protein isolated from the tissues of cold-exposed voles had approximately the same specific radioactivity as protein from warm-exposed voles. Incorporation into protein of small intestine and liver of warm-exposed winter voles was greater than that in summer voles, but cold exposure reduced incorporation into liver, muscle and small intestine, to summer levels. Incorporation of ^{14}C amino acids into brown fat increased in both cold and warm-exposed winter voles, relative to summer voles.

Cold exposure in summer depressed the incorporation of ^{3}H-thymidine into DNA of liver, small intestine and muscle. Specific radioactivity of the DNA in winter voles was not significant-

ly affected by temperature, and was at a similar level to that of cold-exposed summer voles. An exception occurred in brown fat where cold exposure increased specific radioactivity both in summer and winter. These data suggest a block in DNA synthesis during cold exposure in summer and at both temperatures during the winter, in all tissues except brown fat.

The observed changes in specific radioactivities in response to environmental conditions are discussed in relation to the achievement of homeostasis during cold exposure and seasonal cold acclimatization.

INTRODUCTION

Studies of cold acclimation and acclimatization have centered largely on heat balance. Cold-exposed mammals increase their heat production by non-shivering and shivering thermogenesis (Hart et al., 1956; Sellers, 1957; Jansky, 1966). There can also be an increased capacity for heat retention due to increased pelage insulation (Sealander, 1951; Hart and Heroux, 1953).

Increased heat production by non-shivering thermogenesis largely involves brown adipose tissue, but other tissues appear to be involved as well, for example, muscle, liver and kidney (Hannon and Vaughan, 1960; Chaffee et al. (1963). The exact mechanisms are still unclear; presumably they include a reorganization of cellular metabolism, particularly energetics, during cold thermogensis. These changes, in turn, may influence or involve protein and DNA synthesis. A number of studies have dealt with changes in cellular protein and DNA after cold exposure. For example, Gordon and Nurnberger (1955) exposed rats to acute cold (temperature not indicated) for one hour and measured the changes in protein and nucleic acid content of the liver and supraoptic nuclei of the hypothalamus. The assays, when compared with those of the control groups ($76^{\circ} \pm 2^{\circ}F$) showed that the concentration of cytoplasmic protein of both liver and supraoptic cells increased under

the following conditions:

a) brief cold exposure in the intact, fed animals

b) 18-24 hour fast in the intact animal

c) bilateral adrenalectomy in the fed animal.

An increase in total homogenate protein was also found in the microsomal and supernatant fractions of cold-exposed liver. The data also suggested a decrease in concentration of total organ content of nucleic acids following cold exposure of fed and fasted intact animals. Harrison (1953) found a slight increase in total DNA and a decrease in total protein in fasted animals at the same temperature as controls. Trapani (1960) and Wilson (1966) chronically exposed animals to cold and found an increase in total mass of circulating protein, and an increase in protein turnover in the blood. This increase in protein was also found in cold as opposed to warm acclimated monkeys by Chaffe et al. (1966) in the liver, pancreas, heart and kidney. Total DNA (μg/organ) was also increased, and this presumably represents cell proliferation since most of the above organs increased in size when the animals had been acclimated to cold. Somberg and Frascella (1969) showed a decrease in total liver DNA in rats fed a low protein diet at 4°C as compared to those at 25°C. This was also true for rats fed a high protein diet. Petrovic (1969) exposed rats to -2°C for 96 hours and found that

protein and RNA are increased significantly while total DNA remained relatively constant in the adrenal, showing cellular hypertrophy. Chauhan et al.(1969) have demonstrated this increased protein production in the liver, in response to cold, from as early as three hours after exposure to 0-4°C.

A systematic study of protein and DNA synthesis in relation to cold thermogenesis is required to achieve a more complete understanding of fundamental aspects of thermogenesis. The purposes of this study were three-fold: (1) To determine the effect of cold exposure on protein and DNA synthesis in various tissues; (2) To determine the effects of cold acclimatization on protein and DNA synthesis in various tissues; (3) To determine whether cold acclimatization has any effect on (1).

The meadow vole (Mictrotus pennsylvanicus), a wild species, was chosen for this study in order to permit an analysis of natural acclimatization to low temperatures.

LITERATURE REVIEW

A number of mechanisms have evolved in homeothermic animals for the maintainance of a constant body temperature. These involve adjustments such as changes in body weight, insulation, organ weights, metabolite concentration as well as physiological changes, eg. circulation of hormones, increase in size of endocrine glands and increased oxygen consumption.

It has been observed that in cold acclimated mammals, at temperatures below $0^{\circ}C$, weight loss occurs steadily. This loss is greater in animals previously exposed to cold (Hart and Heroux, 1956). At temperatures from 0°-$5^{\circ}C$ for rats, and above $5^{\circ}C$ for guinea pigs, there is an initial loss in body weight during the first ten days, but as the food intake is increased, the animals begin to gain weight slowly (Sellers and Young, 1954; Young and Cook, 1955; Baker and Sellers, 1957). At temperatures above $5^{\circ}C$ but below $20^{\circ}C$, rats and mice do not lose weight initially but grow at a slower rate than controls (Heroux and Hart, 1954; Biggers et al., 1958). This loss in weight has been shown to be mainly due to the utilization of fat and protein reserves (Hart and Heroux, 1956). There is also a decreased deposition of protein in muscles (Heroux, 1958).

The initial decrease in body weight at temperatures below $5^{\circ}C$ is overcome after about one week in the cold when the animal starts

to gain weight slowly. Along with this increase in body weight, there is an increase in weight of certain organs following cold acclimation (Heroux and Hart, 1954; Heroux and Gridgeman, 1958). These authors showed an increase in weight of the liver, heart, kidney and intestinal tract. Knigge et al. (1957) also showed an increase in weight of the adrenal and thyroid glands. The increase in the adrenal weight of the rat is due to an increase in cell size (Petrovic, 1969). The hypertrophy of the adrenal remains constant in the cold even though the initial increase in the hormone level returns to normal. Heroux and Schonbaum (1959) showed that in indoor cold conditions, this hypertrophy was due to an increase in the number of cells in the zona-fasiculata. However, under outdoor cold conditions the adrenal weight as well as the number of fasiculata cells remained normal.

Cold acclimation reduces the growth of other tissues such as muscle, fat depots, spleen, thymus as well as pelt (Heroux and Campbell, 1959). While the white fat was decreased, brown fat in the interscapular pads hypertrophies (Roberts and Smith, 1967). It has also been shown by Himms-Hagen (1969) that the metabolism of brown adipose tissue is increased in the cold as indicated by increased incorporation of glucose-U-^{14}C into lipids of brown fat. It was suggested that this increased incorporation might provide

some measure of the rate of triglyceride synthesis. Cortical steroids released from the hyperactive adrenals is said to increase the fat free dry content (i. e. cells containing mitochondria), water content and the fat content of brown adipose tissue (Lever and Chappel, 1958). The relative abundance of electron transport components such as cytochrome oxidase in brown fat (Barnard et al., 1969) would indicate a functional role of this tissue in utilizing substrate for heat production during prolonged exposure (Smith and Hoijer, 1962). An increased deposition of white fat in the liver has been reported by Vaughan et al.(1958) and is interpreted as a mobilization of fat from body stores, to meet the demand for energy.

Three categories of lipids are affected by cold and hormones. They are: (a) Triglycerides used in the production of heat and high-energy compounds, (b) cholesterol -- important for formation of corticosteroids, (c) Phospholipids -- components for functional mechanisms.

(a) Cold exposure leads to an increased turnover of the triglyceride component for substrate utilization, and this is facilitated by an increase in the phospholipid content of adipose tissue cells (Page and Babineau, 1950). Mobilization of these fatty acids is increased in the presence of nor-epinephrine (and epinephrine) to a small extent which then can be utilized for substrate oxidation (White and Engel, 1958).

(b) Cholesterol level is increased in the plasma by throxine and epinephrine. Both of these hormones are increased in the plasma in the cold (Shafrin and Steinberg, 1960).

(c) Phospholipid production is increased in the cold, in brown fat, liver, kidney and adrenals. It has been suggested that an increase in phospholipid would facilitate an increased oxidation of triglyceride fatty acids (Astrom and Swanson, 1951).

Non-shivering thermogenesis is located in the muscle as well as in brown adipose tissue and liver, but according to Jansky (1966), the thermogenesis in the liver has no apparent relation to cold adaptation in the rat.

As mentioned above, cold acclimation hypertrophies the adrenal and thyroid glands. The increased production of the corticosteroid hormones contributes to the increase in size of the adrenal (Crane et al., 1958). One of the significant functions of these corticosteroid hormones is that of facilitating the production of keto acids from proteins for oxidation in the production of heat (Smith and Hoijer, 1962). This accounts for a 50% rise in protein turnover in cold-exposed animals (Trapani, 1960). This deamination associated with amino acid oxidase occurs principally in the liver and the nitrogen released forms an adequate pool for the production of essential compounds, eg. purines and pyrimidines for co-factor formation (Smith and Hoijer, 1962).

Increased thyroxine production on the other hand tends to accelerate the incorporation of amino acids into proteins (Sokoloff et al., 1960) which tends to counter-balance the effect of corticosteroids on protein catabolism. Thus during cold exposure these hormones enhance the incorporation of valine ^{14}C and acetate-1-^{14}C into protein (Wilson and Siperstein, 1959). In cold-stressed animals, thyroxine also enhances the increased production of creatine phosphate, which is used for energy production in the muscle.

The liver, which is thought to be a major site of heat production in the cold acclimated animal, shows an increased metabolism by increasing the specific activities of a number of glycolytic enzymes e g. glucokinase and glucose-6-phosphate (Hannon, 1960; Hannon and Vaughan, 1960). This suggests a dependence of the cold acclimated animal on the oxidation of hepatic glucose in the production of energy. It has been suggested that the different activities of different enzymes in cold acclimation could be a result of the length of exposure to cold, the age, strain and diet of the animals (Smith and Hoijer, 1962).

It has been shown by several authors that in the more natural outdoor conditions of temperature, light and humidity, rats respond differently to cold than they do under artificial conditions in the laboratory (Heroux et al., 1958; Heroux and Campbell, 1959; Heroux,

1962). For example, they adapt to cold in the winter by producing heat by non-shivering thermogenesis (as in the case of animals acclimated in the laboratory), but have a greater sensitivity to nor-epinephrine (Heroux, 1962). They do this, however, without increased thyroid activity, i.e. there is no hypertrophy of this gland and these "natural" animals show a reduced heat loss because of a lower basal metabolism, greater peripheral vasoconstriction and better fur insulation (Sealander, 1951; Rigandiere and Delost, 1966).

Growth has also been shown not to be affected in the animal as a whole, in carcass weight or muscle weight in wild rats, i.e. no protein reduction (Hart and Heroux, 1963) and also there is no reduction in fat deposition, but rather an increase, during winter.

The hypertrophy observed in the liver, kidney, thymus, adrenals, pituitary, and thyroid of cold acclimated animals was not seen in wild rats living in the natural environment during winter (Heroux and Campbell, 1954). The weights of these viseral organs and endocrine glands were the same during summer and winter. It would thus seem that these changes are not necessary for the survival of animals at low temperatures in the natural environment and most important, that seasonal and laboratory induced changes are not identical in these animals (Hart, 1960). It was further

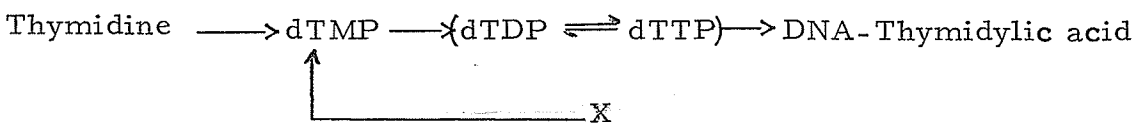
suggested by Heroux (1970) that the hypertrophy of organs seen in cold acclimated animals is a pathological condition brought about by severe conditions when these animals are exposed to cold. These animals are therefore not truly adapted as animals under natural conditions.

In the winter acclimatized animal, brown fat is considered to be a major site of heat production. This heat is produced by the oxidation of fat in the brown adipose tissue (Dawkins and Hull, 1964). Ball and Jungas (1961) have also put forth the theory that heat is produced from the hydrolysis and re-esterification of fatty acids in the brown fat cell. It was shown by Didow and Hayward (1969) that the relative mass of brown adipose tissue of the vole at any one body weight was twice as great in December (winter) as in the summer months. The increase in size of brown adipose tissue could be due to a proliferation of the tissue itself and coincided with a drop in environmental temperature. These changes, related to environmental temperature, were also found by Gilbert and Page (1968) in acclimated white rats, by Aleksiuk (1971) in the red squirrel (Tamiascuirus hudsonicus), and Aleksiuk and Frohlinger (1971) in the muskrat (Ondatra zibethica).

In most biochemical studies involving the incorporation of thymidine into DNA, the tritiated form of thymidine is used. Tritiated thymidine is readily absorbed and distributed after intraperitoneal

injection and its use as a tracer has been established (Reichard and Estborn, 1951). Hughes et al. (1964) showed that 5-^{131} iodo-2^{1} - deoxyuridine (^{131}IUDR) is incorporated into the DNA of rapidly proliferating tissue. Leblond et al. (1959) also showed that labelled nuclei appeared in most tissue (except muscle) immediately after injection of tritiated thymidine. Friedkin et al. (1956) used thymidine-^{14}C to show that the compound was incorporated into DNA as thymidine of proliferating embryonic and animal tissues with little being incorporated into other components of DNA or into RNA. Rubini et al. (1959) state that ^{3}H-thymidine comes into equilibrium with the tissue and is either incorporated into DNA or degraded to tritiated water and other waste substances. These waste substances are not further incorporated into DNA in significant amounts.

It has generally been accepted that in vitro thymidine triphosphate (dTTP) is the precursor of DNA (Kornberg et al., 1956). Potter et al. (1963) tried to show this relationship in vivo using the scheme:



where X represented endogenous precursors of dTMP. They found a rapid equilibration between dTDP and dTTP, and either compound could be a precursor of DNA. Unfortunately, they were not able to reach a conclusion about the mechanism of DNA synthesis in vivo.

Zamecnik et al (1954) have shown that only L-amino acids naturally occuring in proteins are incorporated into animal proteins. The incorporated amino acids are not removed by fat solvents or hot TCA which are used in the extraction of proteins. There is no competition between various amino acids in vitro; instead, there is an additive labelling of the protein. Borsook (1950) showed that labelled amino acids incorporated into a protein are bound in such a way that either their carboxyl or amino groups are involved in the peptide bond. ATP is necessary for this conversion.

MATERIALS AND METHODS

A wild species, the meadow vole (Microtus pennsylvanicus) was chosen for this study to permit the examination of cold acclimatization under field conditions. This species is non-hibernator and thus can be sampled throughout the year.

Approximately fifty voles were collected from their natural habitat (Pinawa, Manitoba) in July, 1970 and December, 1970, and were transported live to the University of Manitoba. Sexual distribution was random, and the voles were considered on the basis of their weight to be juveniles (Table I). The voles were transferred to individual cages, and kept at room temperature ($20^{\circ}C$) overnight. Water only was provided. The animals were starved to empty their intestinal tracts and to eliminate the specific dynamic action of food on metabolism.

Temperature Exposure Studies

Following 16 hours of food deprivation, one group of animals was held at a temperature of $27^{\circ}C$ while another group was held at $15^{\circ}C$ (Table I). Animals were injected intraperitoneally with ^{3}H-thymidine (Sp. Act. 18.35 Ci/mM) and a mixture of ^{14}C-amino acids (specific Activity ranging from 87.4 mCi/mM to 375.0 mCi/mM) in doses of 2 μCi/10 gm body weight, and 1 μ Ci/10 gm body weight

respectively. Injections were given between 10:00 a.m. and 11:00 a.m. Animals in both groups were killed, by ether, at 1/2, 1, 2, 3, 4 and 6 hours after injection. Four animals were killed at each time period (with some exceptions) and quickly dissected. The liver, skeletal muscle from the thigh, small intestine and brown fat were excised and frozen immediately. Samples of blood were also taken into heparinized tubes from the heart of each animal.

Extraction and Measurement of Protein and DNA

The extraction of protein and DNA was done by a modification of the Schmidt-Thanhauser Method (Wannemacher et al., 1965). Samples of the various tissues were homogenized in cold distilled water to a 20% w/v solution. Unlabelled amino acids and thymidine were added as carriers to the homogenate in amounts approximately 100 times the concentration of the injected labelled precursors. Cold 10% (w/v) TCA (8.0 ml) solution was added to 1.0 ml aliquot of the of the homogenate. The precipitate formed was separated by centrifuging at 2000 g for 10 minutes. The precipitate was washed twice with 5.0 ml of cold 10% TCA. Lipids in brown fat were removed at this point by extracting the precipitate with 5.0 ml of 95% ethanol solution saturated with sodium acetate; 5.0 ml of a 3:1 ethanol:ethyl ether mixture and 5.0 ml of anhydrous ethyl ether.

While the precipitate was still moist, 4.0 ml of 0.3 N KOH solution was added and the samples were incubated 60 min at 37 ± 2°C in a water bath. Protein concentration and protein radioactivity were determined on the resulting KOH hydrolysate. Protein concentration was determined using the Buiret method (Wannemacher et al., 1965). A standard curve was established using crystalline bovine serum albumin. In the range 1.0 mg to 8.0 mg of protein per ml KOH solution, the graph produced obeyed Beer's Law.

For the extraction of DNA, 1 ml of 60% (w/v) perchloric acid (PCA) was added to 2 mls of the KOH hydrolysate. The samples were centrifuged for 10 minutes at 13,000 g. The precipitate was washed with 5% PCA, 1.5 ml 0.5 N PCA was added, and the samples hydrolysed in an oven at 96 ±1°C for 45 minutes. The resulting supernatant was used for DNA determinations using the diphenylamine reaction (Burton, 1956). In the range 0.01 mg to 0.04 mg DNA per ml PCA extract, the absorbance obeyed Beer's Law.

Extinctions for protein and DNA concentrations were measured at 540 mμ and 600 mμ respectively, with a Bausch and Lomb "Spectronic 20".

Radioisotope Counting

Aqueous solutions of protein and DNA (up to 0.5 ml solution) were added to 10 ml scintillation cocktail. The cocktail was composed of

0.7% PPO, 0.036% POPOP, 10 ml BBS-3 and 90 ml toluene (Ashton et al., 1970). Mixtures of ^{3}H and ^{14}C were counted concurrently in a Packard Tri-Carb liquid scintillation spectrometer (Model 3320) with red and green channels at pulse height settings of 10-1000 (60% gain) and 100-1000 (4.5% gain). Efficiences as determined with ^{3}H-hexadecane and ^{14}C-hexadecane were 40% and 1% in the red and green channels for ^{3}H and 61% and 74% for ^{14}C.

Quenching was tested for with ^{3}H and ^{14}C labelled n-hexadecane as internal standard

Blood in heparinized tubes was centrifuged in a microcapillary centrifuge. The resulting plasma was weighed and then transferred into scintillation vials to be counted.

The chemicals used were as follows: 2-5 diphenyloxazole PPO and 1, 4 bis 2-(5 phenyloxazolyl-benzene (POPOP) (Fraser Medical Supplies Ltd., Vancouver, B.C.); Bio-Solv solubilizer (BBS-3) (Beckman Instruments Inc., Toronto, Ontario); L-amino acid ^{14}C (u) Mixture (various Sp. Act.), thymidine-methyl ^{3}H (18.35 Ci/mM) (New England Nuclear, Boston, Mass.); n-hexa decane - 12 ^{3}H (2.45 μCi/gm), n-hexa decane - 1 - ^{14}C (1.06 μ Ci/g (supplied by Dr. J.C. Jamieson).

TABLE I

WEIGHTS, SEX AND SAMPLE SIZES OF VOLES

Treatment	Mean Body Wt. (gms)	Sex	
		male	female
Summer 27°C*	33.60	15	8
Summer 15°C**	30.40	16	14
Winter 27°C	17.10	13	6
Winter 15°C	17.62	12	12

* 27°C - warm-exposed voles

** 15°C - cold-exposed voles

RESULTS AND DISCUSSION

Radioactivity of Injected Isotopes in Plasma

Tritium

Summer voles exposed to cold (15°C) had a higher tritium activity in the blood plasma than similar voles exposed to 27°C (Fig. 2). However, using the students T Test at the one hour time interval, this difference was not statistically significant (Table III A). In winter, although only a 90% significant difference was obtained between the warm-exposed and cold-exposed voles for tritium activity in the plasma, the difference was more pronounced than in the summer voles (Fig. 1).

Several workers (Chang and Looney, 1965; Blenkinsopp, 1968; Rubini et al., 1970) estimated ^{3}H-thymidine and its metabolic products in blood. Chang and Looney (1965) estimated that 12.6% of the total tritiated thymidine was in the blood. They found the plasma clearance of this radioactivity was exponential and largely complete one hour after injection. The rate of disappearance of the tritiated thymidine from the blood was found by these workers to be 0.4 μci/min in the first ten minutes and ten times less for the period between 10 min and one hour. Therefore there is a rapid clearance rate of the injected tritiated thymidine from the blood.

In summer, cold-exposed voles had a higher ^{3}H activity in the plasma than warm-exposed voles. This possibly reflects a faster uptake of the tritium from the blood of warm-exposed voles as compared to cold-exposed voles. In winter, the cold-exposed voles had a faster rate of clearance than warm-exposed voles as indicated by the slope of the curve. As in the summer voles, the specific radioactivity of ^{3}H in the plasma, is higher in the cold-exposed voles than in the warm-exposed ones.

In winter there is a higher ^{3}H activity in the blood of cold-exposed voles than in similarly treated summer voles (maximum activity 364 mμ Ci/gm plasma in winter and 276 mμCi/gm plasma in summer). There is a slightly higher ^{3}H activity in the warm-exposed blood plasma of winter voles than summer voles.

^{14}C Activity

Cold exposed summer voles had a higher ^{14}C activity in blood plasma than warm-exposed summer voles (Fig. I). This higher plasma activity in $15^{o}C$ exposed animals may be due to the presence of ^{14}C labelled proteins since it has been shown by several workers (Gordon and Nurnberger, 1955; Trapani, 1960; Chauhan et al., 1969) that protein concentration is increased in cold-exposed animals. In winter cold-exposed voles, the ^{14}C activity was slightly lower in the plasma compared to warm-exposed voles.

In summer, the radioactivity in the blood is slightly higher