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

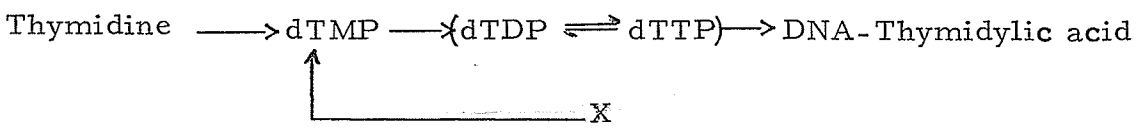
suggested by Heroux (1970) that the hypertrophy of organs seen in cold acclimated animals is a pathological condition brought about by severe conditions when these animals are exposed to cold. These animals are therefore not truly adapted as animals under natural conditions.

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

than in winter. The difference in the slopes of the curves show a faster rate of clearance of ^{14}C activity from the blood plasma of winter voles. The present results suggest this may be due to a slightly greater incorporation of ^{14}C amino acids into protein, in the winter.

Incorporation of ^{14}C Amino Acids Into Protein

Temperature Effects

Incorporation of ^{14}C amino acids into protein in warm and cold-exposed voles did not differ significantly for small intestine, liver and muscle (Fig. 3 and 4). This is in contrast to results obtained by Wilson (1966) and Chauhan et al. (1969) where liver increased its protein turnover at low temperatures. The difference may be explained by the fact that in the present experiment cold exposure was not extreme, so the animals were probably not greatly stressed. Gordon and Nurnberger (1955) and Chauhan (1969) consider exposure to low temperatures a stress which can change organ mass and total protein content.

Brown fat in summer voles showed an increased incorporation of C^{14} amino acids in the cold (Fig. 4). This difference was significant at the one and four hour time intervals. The two hour exposed animals showed a decreased specific radioactivity in both the cold-exposed and warm-exposed animals. Roberts and Smith (1957) found

a decreased respiratory activity and decreased heat production after three hours of cold exposure at $6^{\circ} \pm 1^{\circ}C$ in the interscapular brown fat of rats. They explain this metabolic depression by the fact that the size of the multilocular lipid vacuoles become minimal and hence heat is produced instead by mitochondrial oxidation of exogenous α-ketoglutarate substrate. It follows that this decreased heat production would probably accompany a decreased incorporation of amino acids into protein of brown fat, since any available metabolic energy is probably used for heat production.

In warm-exposed winter voles, there is a significantly greater ^{14}C amino acid incorporation into protein in the small intestine, liver, and muscle than in cold-exposed voles. In brown fat there was no significant change with temperature. The increased incorporation in the small intestine is probably due to growth of new cells, which had decreased in the cold. Since liver and muscle cells do not have a rapid turnover rate, the increased incorporation could be due to a cessation of amino acid catabolism which is used in the production of heat (Wool and Weinshelbaum, 1959) and instead there is increased anabolism, probably due to the action of increased thryoxine (Demarais, 1955). There is little change in the incorporation of amino acids into the protein of brown fat probably because the vole is acclimatized to cold, and since the protein in brown fat may be very important in heat production, the tissue does not immediately

respond to fluctuating temperatures in winter. Had the warm temperature been continued for a longer time, there might have been a reduced protein incorporation since this tissue atrophies at high temperatures and is considered only to be important in the production of heat during cold exposure (Smith, 1964).

Seasonal Effects

In all tissues examined (Figs. 3 and 4), the incorporation of ^{14}C amino acids in the cold in winter, is approximately the same level as incorporation at either temperature in summer. Brown fat is the only tissue where incorporation of ^{14}C amino acids increased in the cold in winter (Fig. 4). This increase in protein incorporation in brown fat is undoubtedly associated with the role of this tissue in heat production (Page and Babineau, 1950; Donhoffer et al., 1964). Jansky (1966) showed that in cold acclimated rats oxygen consumption increased in the muscles, indicating a role of the skeletal muscle in thermogenesis. Heroux (1959) found a decreased oxygen intake in winter out-door rats and Heroux (1961) also found no reduced muscle mass in wild Norway rats. Other factors e.g. no enlargement of the liver, kidney, mediation of nor adrenalin in non-shivering thermogenesis (Heroux, 1962) support the suggestion by Heroux (1961) that winter conditions were not stressful to the animals.

Since there is no decrease in muscle mass in winter wild rats (Heroux, 1961), there should be no decrease in protein content. Therefore the only factor to make a difference in muscle protein turnover in winter, is a decreased metabolic rate. But since the protein specific radioactivity is approximately the same in winter and summer, it might be postulated that there is a compensatory increase in protein metabolism in the meadow vole during winter. There is further evidence of this increased protein metabolism in cold-exposed winter voles from the results presented by liver, small intestine and brown fat (Fig. 3 and 4). It can also be noticed that in the winter voles the slopes of the curves are increased suggesting a higher turnover rate of proteins in winter as compared to summer voles. A similar metabolic change was found by Hart (1953) in deer mice where the limit for survival is extended by 20°C in winter. Since there is but a small change in the insulatory value of fur in smaller mammals (Hart, 1956), the 20°C extension is probably due to a metabolic rather than physical mechanism. Hart (1953) also found no seasonal change in metabolism. Metabolic acclimatization was also found in wild Norway rats by Hart and Heroux (1963) as indicated by: a) Greater elevation of heat production at temperatures below 20°C. b) Greater ability to maintain higher rates of heat production. c) No appreciable difference in oxygen consumption at temperatures in the cold at which metabolism can be

maintained.

In the warm exposed winter voles, ^{14}C amino acid incorporation was higher in small intestine and liver than in summer warm groups. Since these animals were kept at 20°C overnight, this increased protein incorporation may be due to the action of such hormones as thyroxine, growth hormones and steroid hormones. These hormones are known to increase protein synthesis (Korner, 1964; Tata, 1968; O'Malley, 1969). The muscle incorporation was approximately the same in both warm groups, i.e. winter and summer although the rate of incorporation of ^{14}C amino acids seems to be greater in summer while the animal is growing.

Comparison of Incorporation Into Tissues

It is observed that in the incorporation of ^{14}C amino acids into protein, the liver and small intestine were most active of the tissues studied. In the small intestine this may be due in part, to the rapid turnover of cells, as well as the demand for digestive enzymes produced in these cells. Liver produces a large number of proteins, largely in the form of enzymes. These enzymes play a role in the metabolism of carbohydrates, fats and proteins, production of bile salts and detoxification (Nason, 1965). Serum proteins are also produced in the liver. Jansky (1966) showed that the metabolism of liver can be enhanced by the action of such hormones as insulin, by

increased oxygenation of blood and by increased blood flow, with increased levels of substrate in the blood. Some of these conditions can be brought about by exposure to cold, eg. there is an increased blood flow (Hannon et al., 1963) and there is an effect on levels of substrate in the blood (Hannon and Larson, 1962). Due to the nature of these stimuli, Jansky (1966) suggested that there can also be a non-shivering thermogenesis in the liver having no relation to cold adaptation. The high metabolism found in voles (O'Farrel and Dunaway, 1970) will also have an effect on the turnover of protein in tissues like the liver and small intestine. In muscle and brown fat, protein specific radioactivity is about one tenth of that in small intestine and liver (Fig. 3 and 4). The lowest incorporation was seen in the muscle. This agrees with work done by Shenin and Rittenberg (1944) who found that the turnover of muscle protein is much slower than that of the visera. Gordon and Nurnberger (1955) have stated that muscle consists of a small fraction that turns over its protein as rapidly as that of the visera. This protein may explain the incorporation observed in this experiment.

The pattern of incorporation of ^{14}C amino acids into protein observed in this study is taken as an indication of accelerated incorporation of labelled amino acids into protein rather than a slow turnover of the labelled protein. This has been shown by Wilson (1966) and Chauhan (1969). It is further stated by Borsook (1950) that

"accelerated incorporation" may be considered 'protein synthesis' since either the carboxyl or amino acids of the labelled amino acid are involved in peptide bounds. However accelerated incorporation may be due to a decrease in pool size of plasma protein, thus allowing more labelled amino acids to be incorporated.

Incorporation of ^{3}H-thymidine into DNA

Temperature Effects

^{3}H-thymidine specific radioactivity is higher in the liver of warm-exposed summer than in cold-exposed summer voles (Fig. 5). This difference is not significant. This may be because the liver is not an actively dividing tissue. Pelc (1964) has shown that even though mitoses are rare in the liver, significant numbers of cells are labelled, and he suggested that these cells periodically renew their DNA by a process of which details are unknown. Leblond (1950) however, stated that there is a low degree of mitotic activity occurring continuously. The parenchymal cells of the liver are mainly responsible for this fairly permanent, but expanding cell population in adult animals. Therefore, the increased incorporation in the warm summer animals may be due to either increased DNA renewal, or mitotic activity. It must also be remembered that liver cells are polyploid, and even though there is increased incorporation, this does not necessarily mean mitosis occurs.

In the small intestine, incorporation is much higher in warm-exposed than cold exposed summer voles. This increased incorporation is probably due to a higher turnover of epithelial cells. In the summer, when food is available, food consumption is presumably high to permit growth. Therefore, cells in the intestine are lost into the lumen quite rapidly, and there is an increased need to produce more cells, mainly for the production of digestive enzymes. Although incorporation is decreased in the small intestine DNA of cold-exposed summer voles, as compared to that of warm-exposed summer voles, the amount incorporated is still quite high. This might be because in the cold these animals have to eat more food appropriate to the metabolic heat load imposed by cold exposure, (Smith and Hoijer, 1962), and this cold would cause spontaneous proliferation of new cells in the small intestine. The decrease in incorporation may be because a minimal number of cells are now proliferating. Any label in cells that are sloughed off into the lumen is not reincorporated (Creamer, 1961).

It has been shown (Chaffee et al., 1963; Chaffee et al., 1966; Petrovic, 1969) that after cold acclimating animals for several weeks, total DNA increased in the liver and kidney. In the present result, mild cold exposure does not bring about an increase in DNA (as shown by decreased incorporation), possibly because the voles

were not stressed. Therefore, growth is slowed in these organs, the metabolic energy required for cell division probably being used in direct heat production instead. This decreased incorporation of ^{3}H-thymidine into DNA is observed in the skeletal muscle, liver and small intestine (Fig. 5 and 6). It is most evident in the muscle which has been shown to be capable of increasing its capacity for non-shivering thermogenesis (Jansky and Hart, 1963). Therefore what probably happens in this tissue as well as in the liver and small intestine is an immediate "blockage" of cell division, thus allowing this metabolic energy to be used elsewhere in the production of heat.

Brown fat DNA, unlike the other tissues studied, showed increased incorporation of ^{3}H-thymidine in the cold-exposed summer voles. Fig. 6 shows this incorporation increasing with time in summer voles. Roberts and Smith (1967) showed that the total mass of brown fat, relative to body weight increased about 20-25% after three hours in the cold. Brown fat is important in the production of heat in the cold (Smith and Roberts, 1964). Therefore cold per se would probably cause changes in the tissue by other mechanisms (Petrovic, 1969). Woods and Carlson (1956); Cottle and Carlson (1956); and Petrovic (1969) have shown that thyroxine and corticosteroid production are increased in the cold in rats during the summer.

However, these hormones may only have limited action on brown fat (Roberts and Smith, 1967). In the warm-exposed voles, brown fat incorporation of ^{3}H-thymidine is at a steady lower level. This probably means that there is some cell proliferation even in summer. Didow and Hayward (1969) suggested that brown fat is used to produce heat even in summer when the ambient temperature is below the lower critical temperature. This might explain the slight radioactivity in brown fat observed in this study.

The incorporation of ^{3}H-thymidine into DNA of muscle was significantly greater in the warm-exposed as compared to the cold-exposed vole. Enesco and Puddy (1964) have shown that an increased number of fibre nuclei and mitotic figures were observed during growth of rats. It was suggested and subsequently proved by Moss and Leblond (1970) that the nuclei that take up ^{3}H-thymidine and undergo mitosis are the nuclei of muscle satellite cells, and not true muscle cells. The labelled nuclei of the satellite cells are incorporated into true muscle cells about 24 hours after injection. Since the voles used in this experiment were juveniles and therefore would be growing during warm exposure in summer, the ^{3}H-thymidine could be incorporated into the satellite cells, then into the true muscle cells.

In liver, small intestine and muscle, there was a decreased incorporation of ^{3}H-thymidine on exposure to cold. This may be

because the energy for DNA synthesis is now being used in heat production. The liver did not show as drastic a decrease in incorporation in the cold as compared to small intestine and muscle. This may be because cold has no significant effect on the synthesis of DNA in this essentially non-proliferating tissue.

In winter, the differences in specific radioactivity with temperature were small and not significant in the small intestine, muscle and liver. This is probably because the animals are not growing and therefore DNA synthesis is not occurring. Iverson (In preparation) has shown that there is no growth of the meadow vole in winter. Lack of winter growth may be an evolutionary adaptation to low food availability in a cold environment and therefore even when these animals are exposed to warm temperatures, DNA incorporation does not differ significantly from the cold-exposed winter groups (Fig. 5 and 6). This probably represents a block in DNA synthesis in winter, i.e. DNA synthesis is turned off by intrinsic control mechanisms as a result of selection by the environment. In the brown fat tissue, again the incorporation of ^{3}H-thymidine was higher during cold exposure. Didow and Hayward (1969) showed that the mass of brown fat tissue increased during winter. Therefore even in winter the cells of brown fat may be in an active state of cell division. On warm exposing these animals, the amount of ^{3}H-thymidine incorporated was significantly lowered (Fig. 6). Cold

TABLE II

STATISTICAL COMPARISONS OF SPECIFIC RADIOACTIVITIES OF PROTEIN AT ONE HOUR, USING STUDENTS T TEST

A. Effect of Season on Temperature

Tissue	Treatment	T Test Ratios	
		Summer	Winter
Liver	15°C 27°C	-0.4435	3.7705**
Small Intestine	15°C 27°C	-1.4733	2.0196*
Muscle	15°C 27°C	0.6305	3.6703**
Brown Fat	15°C 27°C	1.8917*	0.4111
Plasma	15°C 27°C	0.7137	1.4777

B. Effect of Temperature on Season

Tissue	Treatment	T Test Ratios	
		27°C	15°C
Liver	Summer Winter	-3.7705**	-0.9231
Small Intestine	Summer Winter	-2.2424*	1.3608
Muscle	Summer Winter	-1.3856	1.5241
Brown Fat	Summer Winter	-2.1778*	-0.3151
Plasma	Summer Winter	-0.0646	1.7457

**95% level of significance
*90% level of significance

TABLE III

STATISTICAL COMPARISONS OF SPECIFIC RADIOACTIVITIES OF DNA AT ONE HOUR, USING STUDENTS T TEST

A. Effect of Season on Temperature

Tissue	Treatment	T Test Ratios	
		Summer	Winter
Liver	15°C 27°C	1.3394	0.1118
Small Intestine	15°C 27°C	1.0748	1.5199
Muscle	15°C 27°C	4.3104***	1.7722
Brown Fat	15°C 27°C	1.5434	2.7261**
Plasma	15°C 27°C	-0.7766	-2.2884*

B. Effect of Temperature on Season

Tissue	Treatment	T Test Ratios	
		27°C	15°C
Liver	Summer Winter	3.1470***	3.4621***
Small Intestine	Summer Winter	1.1495	4.0081***
Muscle	Summer Winter	4.3104***	-7.4218
Brown Fat	Summer Winter	1.0254	0.1614
Plasma	Summer Winter	-0.2317	-1.2094

***99% level of significance
**95% level of significance
* 90% level of significance

exposure in summer and winter probably causes DNA synthesis in brown fat tissue. Higher temperatures have an almost immediate effect in reducing the incorporation of ^{3}H-thymidine into the cells of the brown fat tissue.

Seasonal Effects

Cold exposure of the voles in summer reduces the level of ^{3}H-thymidine incorporated to that in winter voles. This is true in all the tissues studied except brown fat. This is particularly evident in the case of the small intestine and muscle, and is slower to occur in the case of the liver. This may demonstrate that the metabolic energy required for DNA synthesis is channeled to a more immediate need, i. e. the production of heat. In the warm-exposed summer voles incorporation by DNA is much higher than cold-exposed summer, or winter voles. This is very evident in muscle and small intestine and may be explained by the fact that the muscle mass of juvenile voles would increase due to growth and also there is higher proliferation of small intestine cells since food is available in summer. In the cold, and in winter, the incorporation of ^{3}H-thymidine is quite high in the small intestine probably due to the increased caloric intake that occurs.

Comparison of Incorporation Into Tissues

Of the tissues investigated, small intestine had the highest incorporation of ^{3}H-thymidine into DNA and this, as stated previously, may be due the rapid proliferation of this tissue. Even in the cold-exposed summer voles and in winter voles the specific radioactivity of DNA is quite high compared to the other tissues. Muscle showed the next highest specific radioactivity probably because of the incorporation of ^{3}H-thymidine into the satellite cells mentioned by Moss and Leblond (1970) in growing mice. Since these were growing voles during the warm exposed summer experiment, this would explain the high incorporation. Cold-exposed summer voles, cold and warm-exposed winter voles had muscle DNA synthesis is approximately equivalent to that in the liver. Liver is considered to be a non-proliferating tissue. Gray et al. (1960) have shown that enzymes such as DNA-polymerase are less abundant in extracts of non-proliferating tissues such as liver and kidney, and thus shows a control of the formation of thymidine tri-phosphate in the regulation of DNA biosynthesis. DNA incorporation in brown fat is low compared to the other tissues, but in summer cold-exposed voles this was increasing with time, possibly due to the proliferation of this tissue in the cold. In December, when the voles were caught for this experiment, their brown fat could have gone through the rapid proliferation and

reached a size where DNA synthesis was at a minimal turnover rate, thereby possibly explaining the low incorporation of ^{3}H-thymidine into DNA in winter.

Small intestine showed the highest incorporation rate in summer and winter compared to the other tissue. Warm-exposed muscle was next highest, but with cold exposure and in winter the incorporation was reduced. Temperature did not seem to have much effect on the incorporation of label into DNA in liver, although in winter incorporation was lower than in summer. Cold exposure increased incorporation in winter and summer brown fat of voles while higher temperatures decreased incorporation of label into DNA of brown fat in summer and winter voles.

Fig. 1 Effect of season and temperature on ^{14}C activity in the plasma of voles at various time intervals after intraperitoneal injection of ^{14}C-amino acids (1 μ Ci/10 gm body weight). Each point plus brackets represent mean ± standard error of values for 4 individuals.

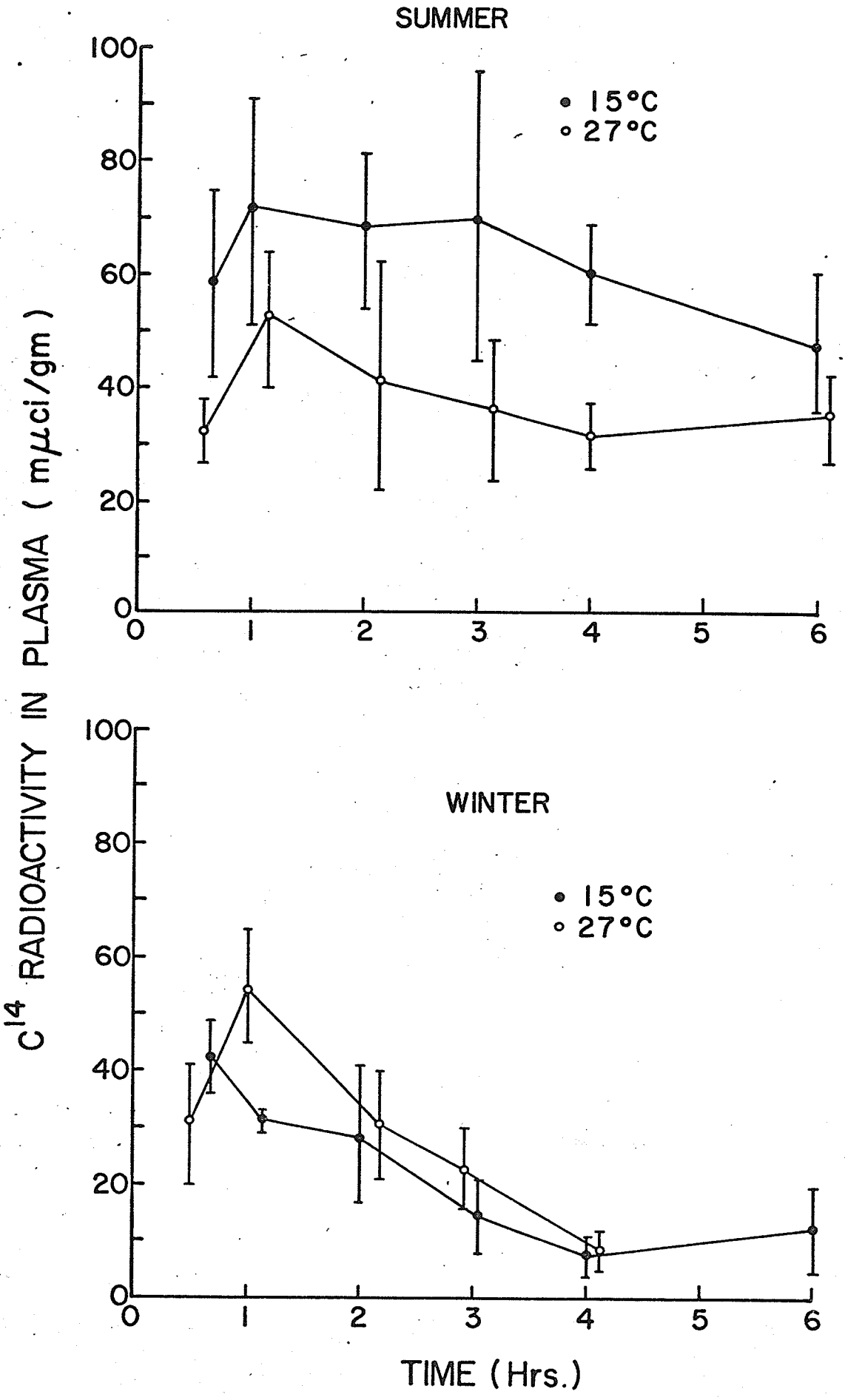

SUMMER
15°C
27°C
100
80
60
40
20
0
0
1
2
3
4
5
6
WINTER
15°C
27°C
C14 RADIOACTIVITY IN PLASMA (mμci/gm)
TIME (Hrs.)

Fig. 2 Effect of season and temperature on tritium activity in the plasma of voles at various time intervals after intraperitoneal injection of ^{3}H-thymidine (2 μ Ci/10 gm body weight). Each point plus brackets represent mean ± standard error of values for 4 individuals.

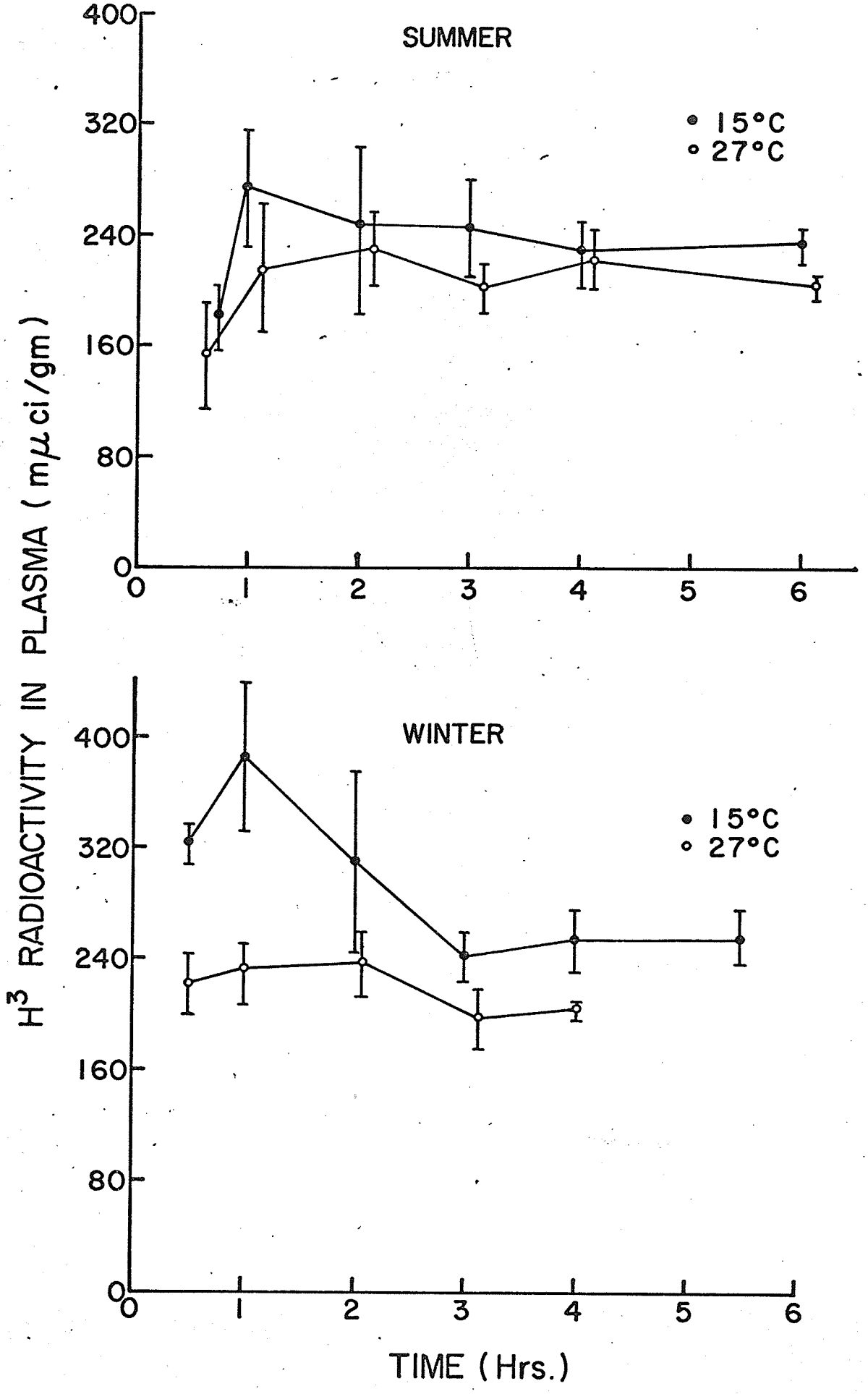

SUMMER
15°C
27°C
400
320
240
160
80
0
0
1
2
3
4
5
6
WINTER
15°C
27°C
400
320
240
160
80
0
0
1
2
3
4
5
6
H3 RADIOACTIVITY IN PLASMA (mμci/gm)
TIME (Hrs.)

Fig. 3 Effect of season and temperature on changes in ^{14}C specific radioactivity of protein from liver and small intestine of voles, expressed as a function of time, after intraperitoneal injection of ^{14}C-amino acids (1 μCi/10 gm body weight). Each point plus brackets represent mean ± standard error of values for 4 individuals.

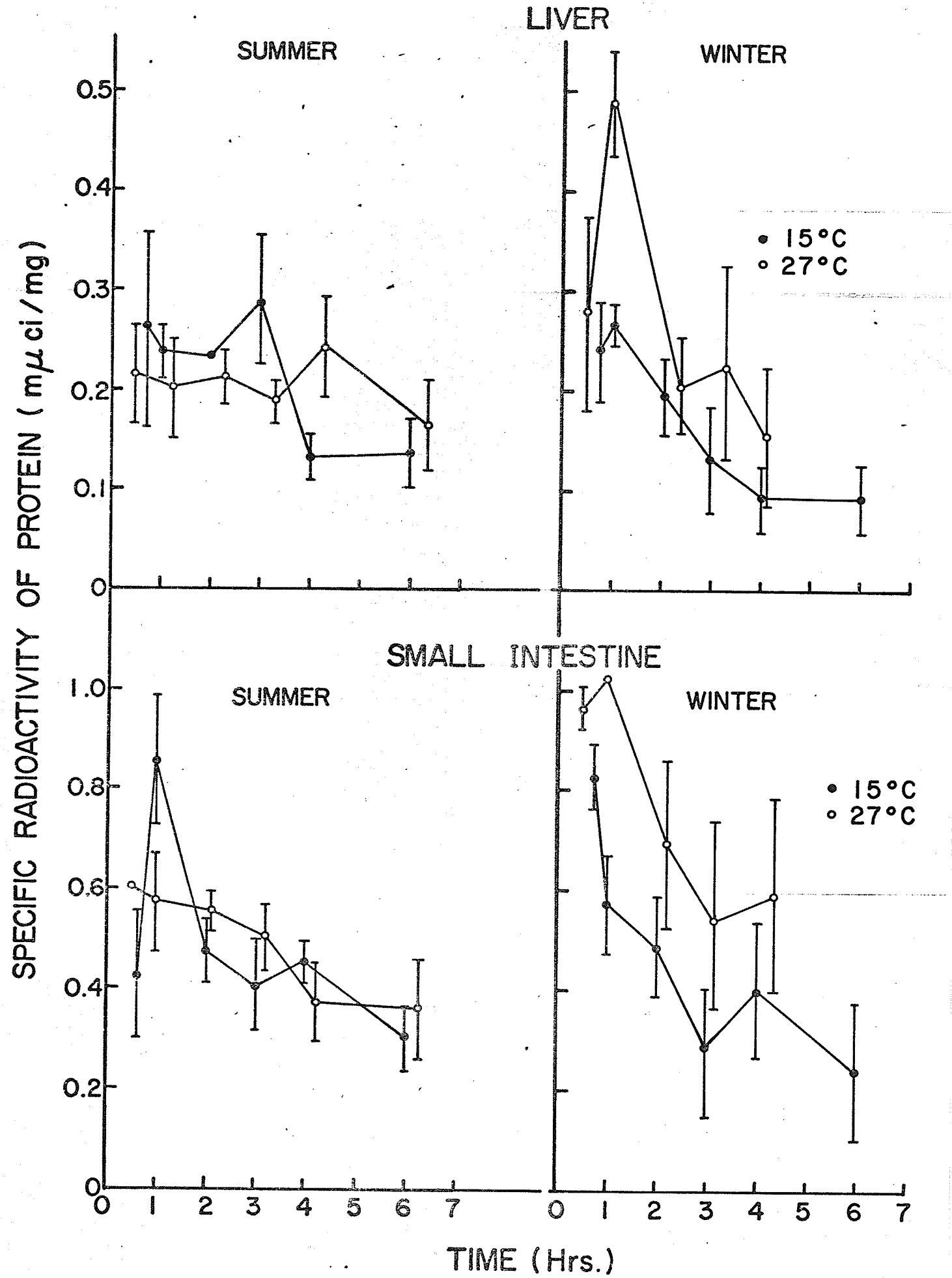

LIVER
SUMMER
WINTER
0.5
0.4
0.3
0.2
0.1
0
• 15°C
○ 27°C
SMALL INTESTINE
SUMMER
WINTER
1.0
0.8
0.6
0.4
0.2
0
• 15°C
○ 27°C
0 1 2 3 4 5 6 7
0 1 2 3 4 5 6 7
SPECIFIC RADIOACTIVITY OF PROTEIN (mμ ci / mg)
TIME (Hrs.)

Fig. 4 Effect of season and temperature on changes in ^{14}C specific radioactivity of protein from muscle and brown fat of voles, expressed as a function of time, after intraperitoneal injection of ^{14}C-amino acids (1 μ Ci/10 gm body weight). Each point (plus brackets represent mean ± standard error of values for 4 individuals.

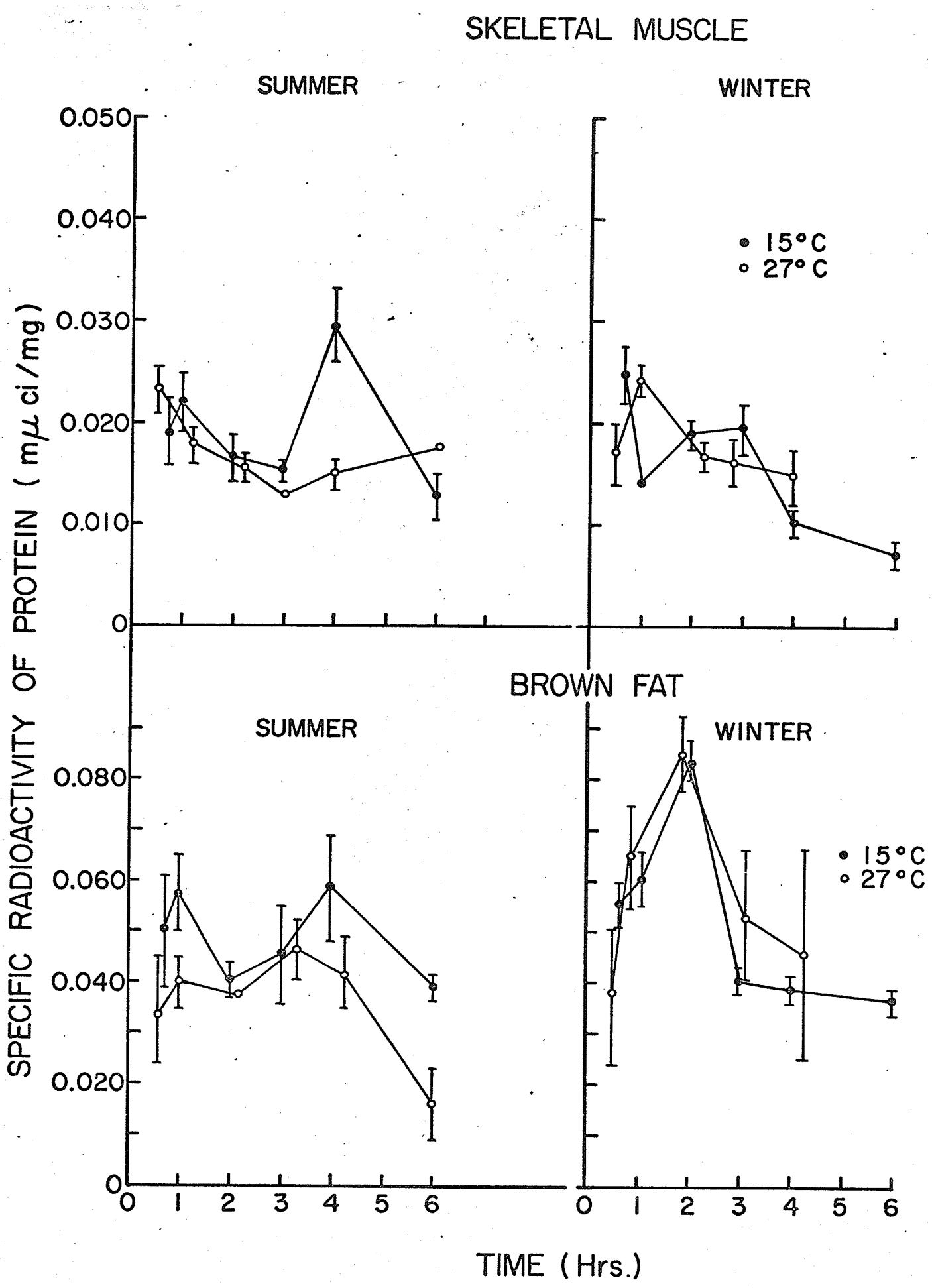

SKELETAL MUSCLE
SUMMER
WINTER
0.050
0.040
0.030
0.020
0.010
0
15°C
27°C
BROWN FAT
SUMMER
WINTER
0.080
0.060
0.040
0.020
0
15°C
27°C
SPECIFIC RADIOACTIVITY OF PROTEIN (mμ ci/mg)
0 1 2 3 4 5 6
0 1 2 3 4 5 6
TIME (Hrs.)

Fig. 5 Effect of season and temperature on changes in ^{3}H specific radioactivity of DNA from liver and small intestine of voles, expressed as a function of time, after intraperitoneal injection of ^{3}H-thymidine (2 μCi/10 gm body weight). Each point plus brackets represent mean ± standard error of values for 4 individuals.

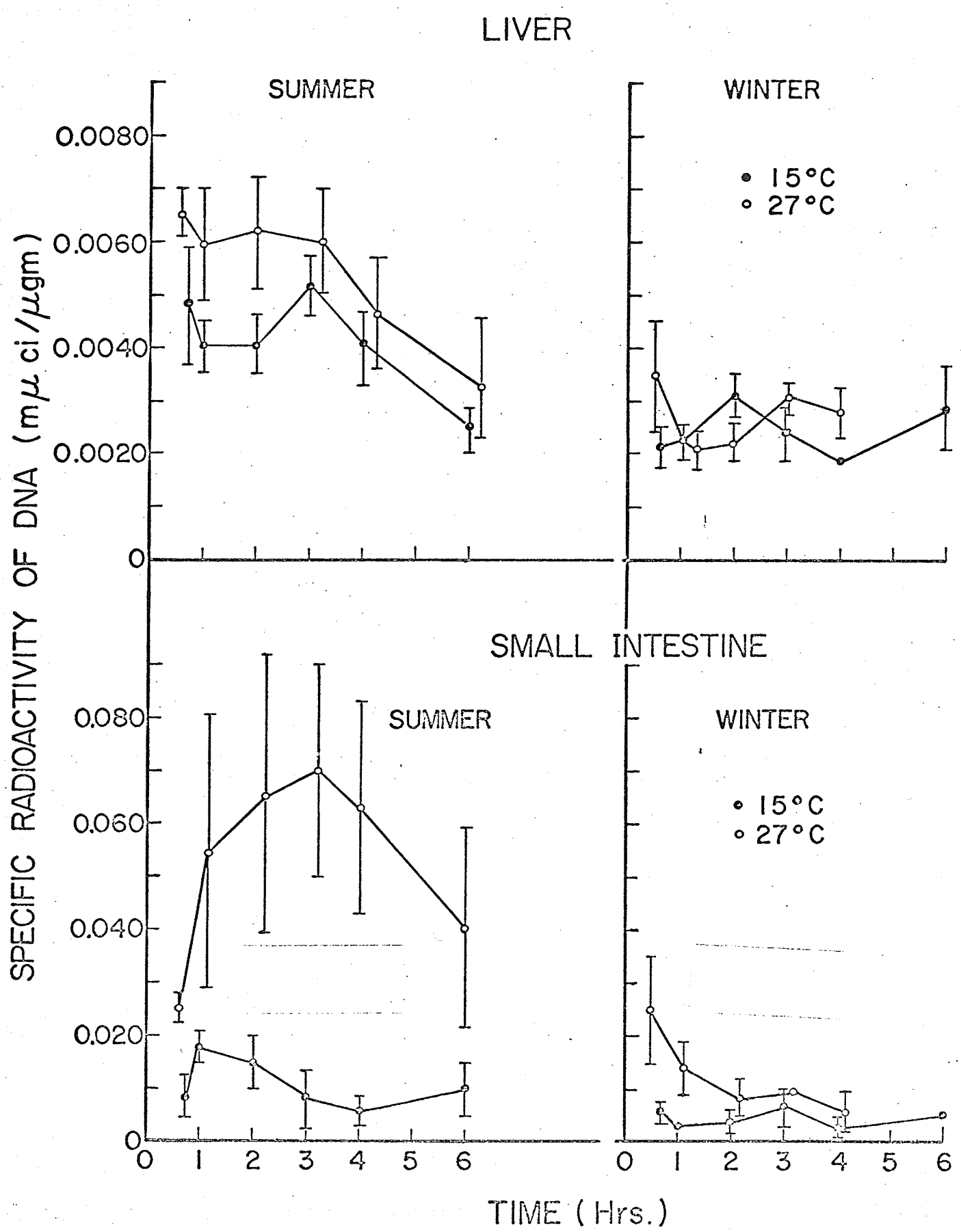
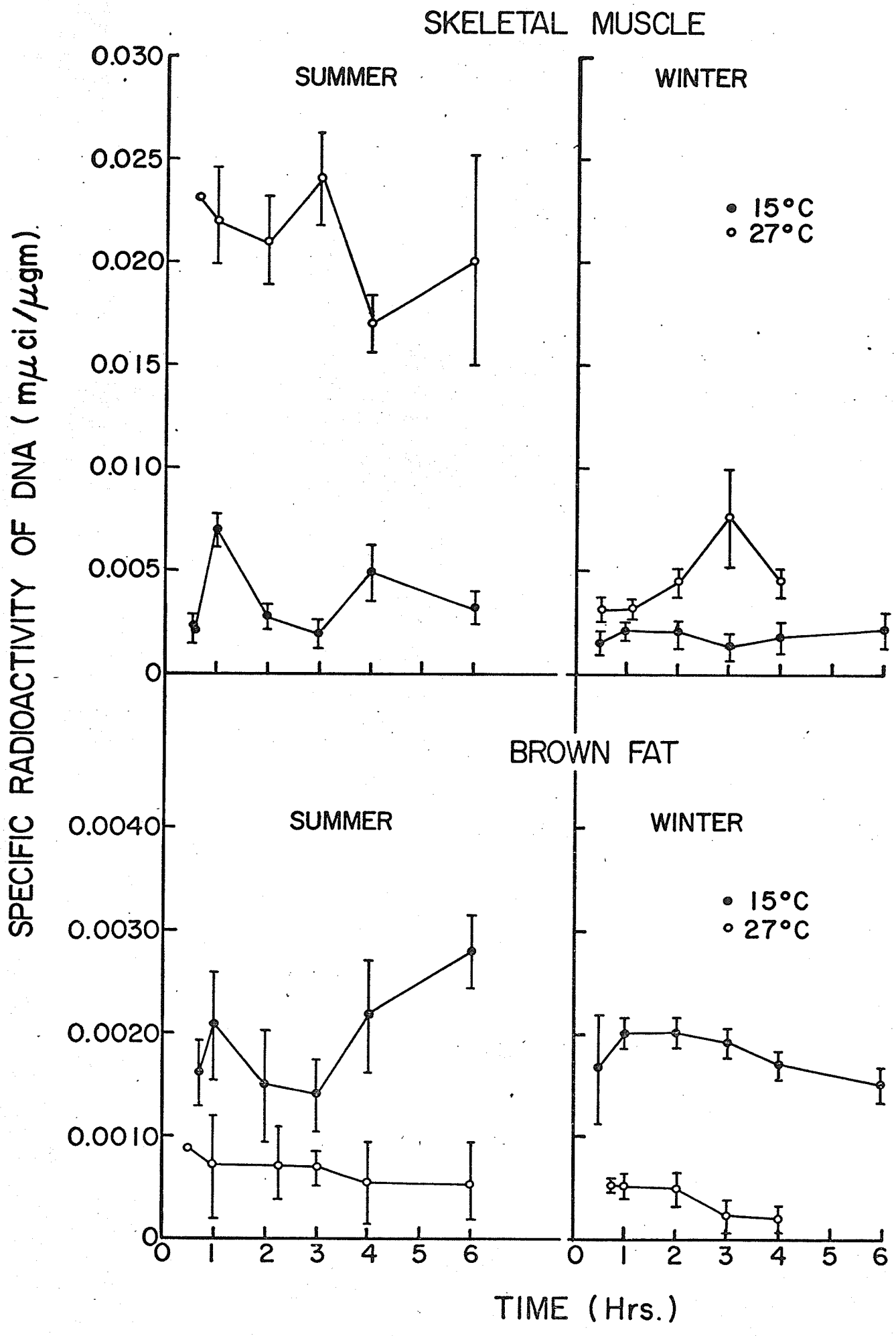

LIVER
SUMMER
WINTER
0.0080
0.0060
0.0040
0.0020
0
• 15°C
○ 27°C
SMALL INTESTINE
SUMMER
WINTER
0.080
0.060
0.040
0.020
0
• 15°C
○ 27°C
SPECIFIC RADIOACTIVITY OF DNA (mμ ci/μgm)
0 1 2 3 4 5 6
0 1 2 3 4 5 6
TIME (Hrs.)

Fig. 6 Effect of season and temperature on changes in ^{3}H specific radioactivity of protein from muscle and brown fat of voles, expressed as a function of time, after intraperitoneal injection of ^{3}H-thymidine (2 μCi/10 gm body weight). Each point plus brackets represents mean ± standard error of values for 4 individuals.


SKELETAL MUSCLE
SUMMER
WINTER
0.030
0.025
0.020
0.015
0.010
0.005
0
15°C
27°C
BROWN FAT
SUMMER
WINTER
0.0040
0.0030
0.0020
0.0010
0
15°C
27°C
0 1 2 3 4 5 6
0 1 2 3 4 5 6
SPECIFIC RADIOACTIVITY OF DNA (mμci/μgm)
TIME (Hrs.)

CONCLUSIONS

1. Summer incorporation of ^{14}C-amino acids into protein is increased very slightly in the cold in muscle and brown fat. This may be associated with the thermogenic function of these tissues. Temperature had little effect on the incorporation of labelled amino-acids into proteins in the small intestine and liver.

2. In winter, cold caused a significant decrease in ^{14}C amino acid incorporation in the liver, small intestine, and muscle. Cold had no significant effect on incorporation in brown fat tissue.

3. The liver, small intestine and muscle tissues of cold-exposed winter voles had approximately the same level of ^{14}C amino acid incorporation as in summer voles. In winter, brown fat incorporated a higher level of labelled amino acids than in summer. Since 15°C is below the lower critical temperature of the meadow vole, the maintenance of incorporation in cold-exposed winter voles probably results from an increased compensatory metabolism in winter.

4. Incorporation of ^{3}H-thymidine into DNA was decreased in the cold-exposed as compared to warm-exposed voles in summer, except in brown fat where incorporation was increased in the cold. The general decreased synthesis in the cold may represent a channeling of energy to heat producing reactions.

5. In winter, ^{3}H-thymidine incorporation was low in all tissues.

Temperature made little difference to incorporation of ^{3}H-thymidine into the DNA of liver, small intestine and muscle. Brown fat of cold-exposed voles had a higher incorporation rate than warm-exposed voles.

6. In summer, incorporation of ^{3}H-thymidine is higher in warm-exposed voles than similarly treated winter voles. Cold exposure in summer decreases ^{3}H-thymidine incorporation to winter levels. Cold exposure caused an increased incorporation in brown fat and this is probably associated with the role of brown fat in heat production.

7. Increased level of protein synthesis, and decreased DNA synthesis during the winter are probably manifestations of homeostatic mechanisms which permit survival under winter environmental conditions.

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