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Energy Sources in Hibernator Heart: Pyridine Nucleotide Metabolism* (33336)

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Previous experiments from this laboratory on the metabolism of hibernator hearts have shown that the pyridine nucleotide metabolism of fluoride-inhibited heart homogenates from bats (*Myotis sp.*) differ markedly from nonhibernator rat heart homogenates (1). Fluoride-inhibited heart homogenates from bats were able to consume oxygen using glucose-6-phosphate as substrate with either nicotinamide adenine dinucleotide (NAD+) or nicotinamide adenine dinucleotide phosphate (NADP+). Rat heart homogenates under the same conditions consume oxygen only with NAD+.

However, the source of energy available to an animal during hibernation and the source of readily available energy for arousal remain obscure (2). Blood glucose levels are lowered and free fatty acids increase during hibernation (3), but the enzymatic means by which hibernator tissue can accomplish an apparent shift away from a glucose-based metabolism to a lipid metabolism in the hibernating state remains largely unknown except in brown fat (4). The present studies offer further evidence that the metabolism of the pyridine nucleotides in tissues from hibernators is altered with the onset of hibernation.

Methods and Materials. Ground squirrels *(Citellus tredecemlineatus)* captured in the vicinity of Chicago, Illinois, were maintained in separate cages throughout the winter months at 4–5° with a 10–14 day-night light cycle. They were fed mouse breeder chow and given free access to water. The animals hibernated readily under this regimen, arousing periodically and reentering hibernation. The average bout of hibernation lasted 12–14 days but some animals hibernated for as long as 10 weeks. Animals were considered for experiments as hibernators after 5 days of continuous hibernation. Aroused, cold-exposed animals were used only if they remained awake for a minimum of 5 days. A total of 54 animals was used in these experiments.

Animals were killed by cervical dislocation.

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Hearts were removed rapidly in the cold, washed in buffer, weighed and immediately minced finely with scissors. The minced pieces were homogenized with 3 ml of *M* 0.067 phosphate buffer, pH 7.4, for 3–4 min in a Sorval microhomogenizer. The homogenized tissue was transferred to a Potter blender with an additional 7 ml of phosphate buffer and blended for 3–4 min. Both homogenizing operations were performed in an alcohol-ice bath and the whole homogenate used without further treatment.

Heart homogenates, 0.5 ml, were added to Warburg vessels containing 0.2 *M* phosphate buffer, pH 7.4; 0.04 *M* nicotinamide; 0.01 *M* magnesium chloride; 5 μmoles of adenosine-5-triphosphate; and 0.04 *M* sodium fluoride (F′); 20% sodium hydroxide was used in the center well for absorbing carbon dioxide. The final volume of each flask was 3.2 ml. Glucose-6-phosphate (G-6-P), 5 μmoles and 0.4 mg either nicotinamide adenine dinucleotide (NAD+) or nicotinamide adenine dinucleotide phosphate (NADP+) were added from the side arm after temperature equilibration. All experiments were carried out in a Gilson differential respirometer at 35°.

Protein concentration was determined by the method of Lowry (5) and the results are expressed as microliters of oxygen consumed per milligram of protein, μl O_2/mg p′.

Results. Control experiments designed to determine the endogenous rate of oxygen consumption showed that without substrate or fluoride, homogenates of 8 hibernating ground squirrel hearts consumed an average of 73 μl O_2/mg p′/hr. With the addition of 5 μmoles of G-6-P and 0.4 mg of NAD+ the rate increased by 116% ($p < .01$) to 158 μl O_2/mg p′/hr. Changing the nucleotide to NADP+ increased oxygen consumption by 104% ($p < .01$) over the endogenous rate. With the addition of F′, all of the oxygen consumption rates were significantly reduced by approximately 50–60% of the fluoride-free rates. Inhibition of oxygen consumption was thus partial rather than complete but the degree of inhibition of any particular pathway cannot be determined from these data because of the variety of enzyme systems possible in the whole homogenate.

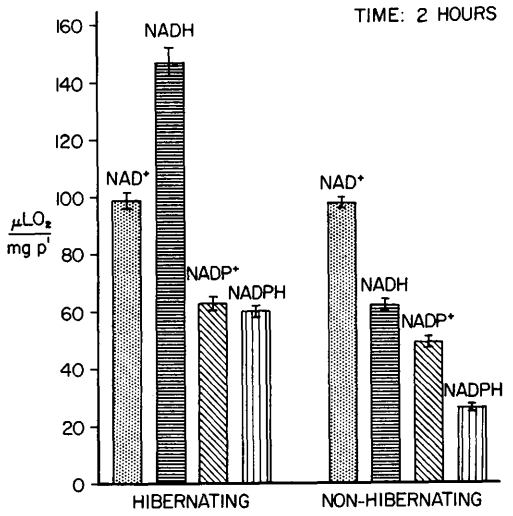

FIG. 1. Total endogenous oxygen consumption over a 2-hr period by homogenates of hibernating and nonhibernating ground squirrel hearts in the presence of 5 μmoles of G-6-P, 0.4 mg coenzyme, and 0.04 *M* F′. Conditions given in the text. Bars represent averages of 8 homogenates, vertical lines indicate ± SD.

The results obtained over a 2-hr incubation period with both oxidized and reduced forms of NAD+ and NADP+ are given in Fig. 1. The bar graphs represent the total oxygen consumption less the oxygen consumption of G-6-P free homogenates. Brei from nonhibernating animals showed total (2 hr) oxygen consumptions of 96 μl O_2/mg p′ and 52 μl O_2/mg p′ with NAD+ and NADP+ respectively. Oxygen consumption with reduced coenzymes was 62 μl O_2/mg p′ and 32 μl O_2/mg p′ with NADH and NADPH over the same period. Different results were found with the homogenates from hibernating animals. The oxidized forms of the coenzymes stimulated oxygen consumption to 111 μl O_2/mg p′ with NAD+ and to 72 μl O_2/mg p′ with NADP+. In contrast to the results with nonhibernator tissue, the reduced forms of the coenzymes stimulated oxygen consumption by hibernator tissue as well or better than the oxidized forms. Reduced NAD+ increased oxygen consumption to 164 μl O_2/mg p′ and reduced NADP+ increased consumption to 66 μl O_2/mg p′.

Table I gives the rates of oxygen consumption by heart homogenates. Homogenates

TABLE I. Average Endogenous Rates of Oxygen Uptake by Homogenates of Hibernating and Nonhibernating Ground Squirrel Hearts in the Presence of 5 μmoles of G-6-P, 0.4 mg coenzyme, and 0.04 *M* F′. Conditions given in the text.[a]

	μl O_2/mg p′/hr ± SD		
Coenzyme	Hibernating	Non-hibernating	% difference
NAD+	50.70 ± 1.8	44.30 ± 3.4	12
NADH	74.60 ± 6.7	30.80 ± 1.6	58
NADP+	31.40 ± 2.2	21.03 ± 6.1	33
NADPH	30.80 ± 2.5	12.46 ± 2.2	56

[a] $N = 8$ homogenates.

from nonhibernating animals consumed oxygen in the presence of oxidized coenzymes at rates that were significantly higher ($p < .01$) than the rates with the reduced forms. By contrast, the rates of oxygen consumption by homogenates from hibernating animals were greater in all cases, particularly with reduced coenzymes. There was a 56% increase in the rate of oxygen consumption in the presence of NADH over that found with NAD+. In all our experiments the rates of oxygen uptake were consistent over the experimental period of 150 min; *i.e.*, no marked changes in rate occurred during any experiment and the results from one experiment to another were remarkably consistent.

Discussion. The inhibition of glucose oxidation by F′, though incomplete under the conditions of these experiments, reduced the activity of magnesium-dependent enzymes at several steps along the glycolytic pathway below the triose level. Fluoride does not, however, affect the operation of the hexose monophosphate shunt or the metabolism of fatty acids. It is thus possible that the mechanisms operating most effectively in our system are those most concerned with the production and use of reduced coenzymes; *i.e.*, the pentose cycle and fatty acid metabolism. It is clear that there are differences between the awake and hibernating states of ground squirrels with respect to pyridine nucleotide metabolism.

Since the nonhibernator hearts show enzymatic activity with oxidized coenzymes that may reflect glycolytic and/or Krebs' cycle activity, it is suggested that cardiac metabolism in the nonhibernator is carbohydrate-based. On the other hand, it is our belief that the increased use of reduced coenzymes by hibernator heart homogenates indicates that the source of energy in hibernation is lipid. It is postulated that with increasing lipid turnover in the hibernating heart there is an increased need to synthesize fatty acids which requires reduced NADP+. Concomitantly, the need for reduced NAD+ for the breakdown of lipid is increased. Thus, the addition of NADP increases the activity of the pentose cycle that leads to the production of NADPH. Our system apparently makes no distinction between reduced NADP+ formed in this way and added NADPH; the end result is the synthesis of fatty acid, which is in turn degraded, perhaps to ketone bodies; *e.g.*, betahydroxybutyrate transhydrogenase step in the latter pathway requires NADH. Such a scheme of concomitant synthesis and degradation of fatty acids in hibernating tissue is implied by the recent studies of hamster brown fat by Prusiner *et al.* (3). Earlier work on fatty acid and glycerol levels in the serum of hibernating hedgehogs showed an increase in esterified fatty acids during hibernation (2).

It seems possible, then, that the control of energy source for cardiac metabolism during hibernation involves the sort of carbohydrate to fat shift demonstrated by Davis and Quastel (6) in fasting animals. Investigations are in progress pursuing this possibility.

Summary. Homogenates prepared from the hearts of hibernating and cold-exposed nonhibernating ground squirrels (*Citellus tredecemlineatus*) have been studied with respect to the metabolism of both oxidized and reduced forms of pyridine nucleotide coenzymes in the presence of fluoride and glucose-6-phosphate. The rates of oxygen comsumption by preparations from nonhibernating animals were highest with the oxidized forms of the coenzymes. By contrast, the rates of oxygen consumption by heart homogenates from hibernating animals were highest with the reduced forms. It is postulated that these findings indicate the operation of a cyclic lipid metabolism in the heart of hibernating

animals that does not operate or is depressed in the aroused state.

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Studies on the Physico-Chemical Inactivation of Rabbit Interferons* (33337)

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In previous communications, we reported that rabbit serum interferons induced by virus and by endotoxin differed in their stability to heat and acid (1, 2). On the basis of molecular size as determined by Sephadex gel filtration, at least two types of virus-induced interferon (VII) and two types of endotoxin-induced interferon (EII) could be identified (2, 3). Both VII and EII contained a minor high molecular weight (>100,000) peak that was designated the "A" peak (VII-A and EII-A). The molecular weight of the main component of VII was 46,000 (VII-B), and that of the main component of EII was 54,000 (EII-B).

In this paper, we report further studies differentiating VII-A from VII-B and EII-A from EII-B on the basis of kinetics of inactivation at 56° and at pH 2. To pursue these differences along other lines, the stability of VII-A and VII-B in the presence of a number of reagents reactive against known chemical radicals, including periodate, was determined.

Materials and Methods. Details on viruses, tissue culture, interferon preparation, and assay techniques were as described elsewhere (2, 4) unless specified. Rabbit kidney cell cultures prepared from 2–3-week-old albino rabbits (about 300 g) were used as we found that they were on the average 8 times more sensitive to interferon than rabbit embryo cultures employed earlier (2).

Serums were obtained from rabbits after inoculation of Newcastle disease virus or *E. coli* endotoxin (Boivin, 2). Virus-induced interferon (VII) was fractionated by filtering 6-ml serum aliquots through a Sephadex G-100 column as previously described. Interferon activity peak regions were pooled and concentrated to 1.5–2.0 ml by dialysis against Carbowax 20 M (polyethylene glycol, manufactured by Union Carbide Corp., South Charleston, West Virginia). These pooled, concentrated semipurified fractions will be referred to as VII-A (m.w. >100,000; interferon activity 1000 units per 2.0 ml), and VII-B (m.w. 46,000; interferon activity 16,000 units per 2.0 ml, protein content 7.5 μg/unit). Endotoxin induced interferon (EII) was similarly processed and concentrated. The resulting EII-A (m.w.>100,000) titered about 400 units per 2.0 ml and EII-B (m.w. 54,000) titered 800 units per 2.0 ml.

For heat inactivation, 2 ml containing about 200 units of a designated interferon

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