FINAL REPORT

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STUDIES ON HIBERNATION-INDUCING FACTOR

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1. Introduction; The Trigger for Hibernation: Hibernation in mammals is the assumption of a state of greatly reduced core temperature instead of the usual temperature of about 37°C; meanwhile the mammal retains the capability of spontaneously rewarming back to the normal homeothermic level without absorbing heat from its environment. There have been some studies on a biochemical factor which initiates this hibernation state. These studies, done at Loyola University, demonstrated a blood-borne substance obtained from hibernating ground squirrels or woodchucks which, when injected into summer-active ground squirrels, induced hibernation. This material is referred to as "hibernation induction trigger" (HIT); it has been shown to be of low molecular weight, is found only in the hibernation state, and can be effective between several species.

2. Work of Dr. Peter Oeltgen in Isolating HIT: Plasma or serum from hibernating animals has been fractionated by Dr. Peter Oeltgen of the University of Kentucky. When these plasma fractions in which albumen predominates, were injected into summer animals, all hibernated, while no control animals hibernated. It is important to note that this hibernation-inducing factor is closely associated with albumen. Dr. Oeltgen has continued the isolation, purification, and characterization of this hibernation-inducing substance. The material is of particular interest to biomedical science because there are very few naturally occurring metabolic-reducing materials. There are applications for the Navy of such metabolic-reducing materials. They might possibly be developed as a fever-reducing agent for sick personnel on the high seas. Or as a substance to reduce metabolic rate of starving populations at the time of famine.

3. Goals: A. To obtain and study the hibernation-inducing trigger (HIT) or factor in woodchucks. B. To maintain in health 14 woodchucks as blood donors. C. To obtain summer control samples of blood by standardized techniques, of woodchucks, hamsters, gerbils, and dogs. D. To obtain blood samples from several species in deep hibernation in the laboratory (woodchucks, hamsters). E. To obtain blood samples from hibernating bears in the field. F. To develop and use a new bioassay for the hibernation-inducing factor. G. To test this factor on rats and monkeys.

4. The Experimental Animals:

Late Winter 1979: In November we transported from Loyola University, 11 woodchucks which have been used in their experiments for several years. There were already three of our own for this project at The University of Iowa, making a total of 14 in the colony. The colony then shrank considerably.

Two animals died in hibernation at the end of the winter of 1979, presumably of old age; a young animal apparently swallowed a piece of wire and died from intestinal perforation. After the hibernation period which ended in April (1979) the three oldest woodchucks were either sacrificed or died during the summer. These had been kept at Loyola for as long as seven years and had been caught in the field in Pennsylvania before that time. They stopped eating and showed variable symptoms such as shivering. Upon necropsy, all three of these animals showed liver pathology. We were then left with a relatively young colony of eight enimals.

Summer 1979: The remaining eight animals were divided; four were kept indoors in small cages and four were maintained in large outdoor cages all summer.

5. Technique: Before the hibernation period, each animal was put under anesthesia with the standardized immobilizer called Innovar. This had been used in the program at Loyola from the start. Cardiac punctures were done and 20 ml of blood was taken and immediately cooled in chipped ice. It was transported to a refrigerated centrifuge, spun, and the plasma was frozen and maintained at -40°C. This technique was satisfactory for providing material to Dr. Oeltgen at the University of Kentucky and Dr. Roberts at Creighton University. Some samples, however, were dialized and both fractions were frozen and maintained. The technique was the same with the hibernating animals in the winter except that Innovar was not needed. This had been the standard procedure in all experiments at Loyola. The plasma was then processed by Dr. Oeltgen, and some was returned to us in powder form (see Appendix A).

6. Non-Hibernating Samples Obtained:

Summer 1979: Our most abundant samples for the first part of the year were obtained from summer animals as controls. We stockpiled or gave to Dr. Oeltgen, 20 10ml samples from woodchucks. We also obtained control samples from hamsters and gerbils. Every woodchuck (N=8) provided a control sample.

Early winter 1980: All animals did not hibernate. A second series of control samples was taken from these non-hibernating specimens. Part of each summer or winter control sample was then provided to Dr. Larry Lutwick to test the plasma for woodchuck viral hepatitis. In all, 26 samples of 10 ml each were taken.

7. Hibernation Success:

Late Winter (Jan., Feb.) 1979: Because of the late start in the hibernating season, very few of the 14 woodchucks hibernated. The animals were first exposed to cold on November 11. The first hibernation occurred soon after that date. Only 4

of the colony hibernated and it is of interest that all 4 of these were females. We attribute the lack of hibernation to the disturbance of being moved from Loyola to The University of Iowa. Three of the 4 woodchucks which hibernated were already being maintained at The University of Iowa at the time the Loyola colony was moved . The details of the move were as follows: On the 27th of October, 1978 the funding of moving the colony to The University of Iowa was informally approved by the Project Officer. Formal approval at that time was anticipated in two months. On the basis of this information, on November 8, this applicant drove a truck to Loyola University and moved 11 woodchucks to The University of Iowa. Formal approval of a contract to maintain these animals and obtain hibernation plasma from them was received on January 1, and the first money arrived at the University on January 31, 1979. This meant a somewhat slow start in establishing the animals in cold rooms for their hibernation period, and we presumed that they were somewhat disturbed by the moving process. Nevertheless, a barely adequate amount of hibernation plasma was obtained from them during the winter.

Early Winter (December) 1980: The second hibernation season was different from the first because 4 animals had been kept indoors in small cages and 4 outdoors in a large dog-run. These outdoor animals could eat grass and enjoy considerable exercise and freedom. The 4 outdoor animals were moved indoors in October and all hibernated; none of the indoor animals did except one for a brief period.

During both hibernation seasons, the procedure was the same. The animals were weighed before and after the season; they were examined twice a day and fur temperatures were taken to prove the condition of hibernation. Cardiac blood samples were taken only after the animal had been in deep hibernation for two days. An example of the sequence of bouts of hibernation is shown for one animal (Fig. 1) and all bouts are recorded in Table 1.

8. Hibernation Samples Obtained:

Late Winter 1979: We did not always obtain perfect samples from hibernating woodchucks. In a few cases hemolysis occurred. Such samples were saved for use with the bioassay. However, 8 separate vials containing 10 ml of clear woodchuck plasma were provided to Dr. Oeltgen the first winter. He would have preferred larger amounts than this, because it is essential in this field of isolation and purification to use fresh material.

Early Winter 1980: Much better success was realized the second winter. About 200 ml of hibernation blood was taken resulting in 80 ml of clear plasma. In March, 40 ml of this and 30 ml of control plasma were sent to Dr. Oeltgen, and small amounts of both were sent to Dr. Jane Roberts of Creighton University.

9. Samples from Other Species: We did not want to limit ourselves to the woodchucks alone and obtained control blood and hibernating blood from both hamsters and gerbils. We also had the good fortune to be in collaboration with Dr. Lynn Rogers of the U.S.D.A. Forest Service at a laboratory in Ely, Minnesota. He was studying the hibernation of bears in northern Minnesota and was to place 6 animals under immobilization by Innovar. Because this was the same immobilizing material that we were using with woodchucks, we took the opportunity to have them prepare samples for us. When the blood samples were obtained from bears, they were immediately covered with ice and taken to a centrifuge in a cold room. The plasma obtained was brought to the University of Minnesota and one of us went there to transport this valuable plasma back to our laboratory. All these samples (hamster, gerbil, and bear) were tested by our hamster heart bioassay. The control was Ringer's Solution. Hibernating hamster plasma was not tried because none of our hamsters hibernated; also we could not obtain summer (non-hibernating) bear plasma, so for a control, we compared hibernating bear plasma with dog plasma.

10. The Bioassay: We decided to try hibernation-inducing factor on newborn hamster hearts. The reason for selecting this heart is that it is very thin-walled and it was not necessary to carry out perfusion through the aorta in order to reach the coronary vessels. There is one disadvantage of this procedure and that is that the hamster has not yet been shown to respond to hibernation-inducing factor. We have obtained the factor from the hamster but have not yet been able to facilitate winter hibernation or produce hibernation in the summer in hamsters. Nevertheless, they are convenient hibernators which may show the effects in the bioassay of a very dilute hibernation-inducing factor. It is necessary to develop a convenient and standardized bioassay because so far the hibernation-inducing factor has been tested in a cumbersome way by injecting ground squirrels in the summer and determining whether they go into hibernation. With this hamster heart assay in pilot studies we found that the newborn hamster heart, when exposed and partially isolated, even without perfusion at room temperature, maintained a rate of 51 bpm (SE 3.4) for 72 minutes (SE 2.8). We then used two procedures; the first was to perfuse the outside of the heart with Ringer's Solution. We then succeeded in cannulating the inferior vena cava of the hearts. There was very little difference in the activity of the hearts when the two methods were compared, presumably because the heart is so thin-walled that perfusate reaches the sinus node from the outside, and oxygen diffuses readily to the cells. Therefore, for this phase of this project, we used only exterior perfusion. The method was tested by using epinephrine in the perfusate and the heart responded very sensitively. After the initial pilot studies, 32 hearts were prepared for formal tests of lactate solution and

the plasma of five species of animals, namely woodchuck, hamster, gerbil, black bear, and dog. The first formal test consisted of using hearts of different weights. The heart rate was slower in the heavier and older hearts (Fig. 2). The hearts lasted longer with one- or two-day-old newborn hamsters than they did with older young, and therefore our test was standardized with young hamsters which weighed 16 ±0.6 mg (SE). In most cases plasma which had been frozen was brought to room temperature and compared with Ringer's Solution control. In one experiment, purified material from woodchucks provided by Dr. Oeltgen was used. More analysis of the effects of the plasma of five species on these hearts needs to be done. One example will be presented in detail, however.

First the method of approach should be described. In earlier experiments, the method of control was to expose the hearts for several minutes to Ringer's Solution and then to experimental plasma for several minutes, and then back to Ringer's Solution. Occasionally a second type of plasma was used after the previous three exposures. After experience was gained, it was determined that the best procedure was to use only two minutes of Ringer's Solution followed by a long exposure to a particular plasma. This could only be compared with extended Ringer's control experiments. Therefore a theoretical curve was designed, based upon six experiments using Ringer's control only, for a prolonged period. This theoretical Ringer's control is found in Fig. 3A and Fig. 3B. Superimposed upon the Ringer's control is the effect of black bear plasma from hibernating animals. Because we have not been able to obtain summer control black bear plasma, we did make the comparison with another carnivore related to the black bears, namely the domestic dog. It can be seen in these results that there is no powerful biological effect of this hibernation plasma upon the newborn hamster hearts.

11. Special Experiments on Rats and Monkeys: In the laboratory of Dr. Gisolfi which adjoins the laboratory of Dr. Folk, experiments were done with a small amount of the purified material provided by Dr. Oeltgen. The hypothalamus was prepared for perfusion in two rats and one monkey. The preparation for the monkey was more elaborate in that the anterior, the middle portion, and the posterior hypothalamus were all cannulated. Experiments were done to perfuse only the anterior hypothalamus in all three specimens. Although there was no effect from the purified material, this experiment will be repeated this summer. with the posterior hypothalamus. We are encouraged to continue these attempts because of the success of Dr. Myers who infused into the third ventricle this same material. He states that he obtained "a clear-cut metabolic action of the trigger." It must be pointed out that it is a very different experiment to expose this material on a part of the hypothalamus and, to infuse it into the third ventricle. on the other hand,

12. Survey for Viral Hepatitis: An interesting spinoff from having this colony of woodchucks has been a survey carried out with Dr. Larry Lutwick of our Department of Internal Medicine. He has been working for several years on viral hepatitis in human subjects and found, as did others, that the woodchuck is the animal model for the study of this disease. Therefore I have taken blood samples from 14 woodchucks under a variety of circumstances such as in summer, in the non-hibernating condition in the winter, and while in hibernation. He has tested all of these samples, which were prepared as plasma in a refrigerated centrifuge, and he is now with a new technique repeating the test on all samples. I will inform the Office of Naval Research about these results when they are obtained. At least we have had the opportunity to compare two types of animals in our colony, those which were obtained from Pennsylvania and those which were obtained from Iowa.

13. Assistance of Co-Investigators: We wish to acknowledge the contributions of members of this team to this project. Dr. Paul Cooper was responsible for the health of this large colony and many problems did appear which he succeeded in solving. Most importantly, on three important occasions when animals in hibernation needed to be exsanguinated, he succeeded in obtaining large quantities of plasma. It is more difficult to do cardiac punctures on these animals when they are in hibernation. The responsible investigator took blood samples of all animals, winter and summer, both for the hibernation factor experiments and for the viral hepatitis study. Dr. Rogers provided the plasma from six black bears in hibernation. We have used only a portion of this material and will continue studying it. Vincent Morinello was responsible for care of the woodchucks when they were out of the formal University animal colony, and conscientiously recorded the degree of dormancy of each animal each day throughout two winters. He has also been most helpful in the reduction of the data.

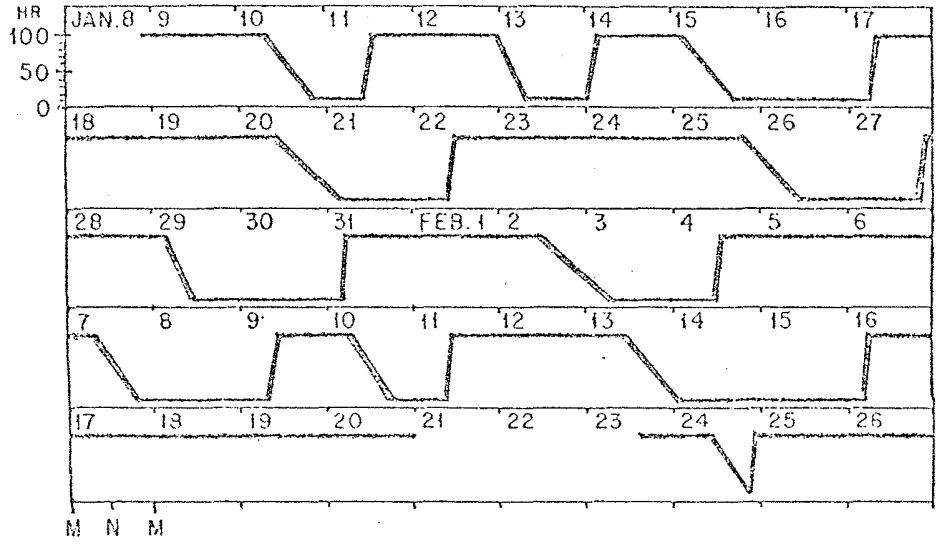
14. Expenditures: It should be pointed out that there was an extension on this contract. Originally it was to end on the first of November, 1979. An extension was provided without additional funds until January 1, 1980. To clarify our expenditure report, I have been in constant communication with our Business Office. It is my understanding that they are forwarding a separate expenditure report to your office.

15. Summary: The purpose of this contract was to provide abundant hibernation and control plasma to Dr. Peter Oeltgen for separation and isolation studies. A second function was to devise a bioassay for detecting hibernation-induction trigger. Both of these goals were met. During the contract period, there were two other accomplishments. A review synthesis of the field was written by the investigator and his student, to relate

blood-borne trigger with brain peptides. This has been a very popular review with about 215 requests for reprints (Appendix B). Also the opportunity finally developed to record earlier data collected under an ONR contract. Several years ago, the responsible investigator traveled to the Naval Arctic Research Laboratory, fasted the two polar bears there for 48 hours (twice) and took blood samples. There were time-consuming analyses done which remained in notebooks, but now with Terry Kaduce and Arthur Spector, we have sent this manuscript to the Journal of Comparative Biochemistry and Physiology (Appendix C).

An overview of hibernation-inducing trigger is now in order. The laboratories which have been supported by the Office of Naval Research should feel grateful for and satisfied by the support provided for this reason: the molecule under study is elusive and delicate and does not have an easily determined powerful biological effect. We did not know this. We have been fortunate in having adequate financial support from the Office of Naval Research to make sure of this. Now it should be the task of agencies of other types (perhaps pharmaceutical houses) to support the difficult experiments of a very subtle type which lie ahead.

Figure 1.



Entering and Awaking from Hibernation. This record of woodchuck hibernation shows cycles of dormancy and the normothermic condition. The heart rate was monitored by implanted Iowa radio-capsule. Ten bouts of dormancy in January and February were recorded. All awakenings were spontaneous.

Table I.

Hibernation in Woodchuck Colony
at The University of Iowa During Winter of
1978-1979 (I) and 1979-1980 (II).

I				II		
Number of Animals			14		8	
Number which Hibernated			4		5 (1 briefly)	
Total Number of Days (for animals which hibernated)		H	153		150	
		S	60		129	
		A	156		121	
Total Days in Cold			106		105	
		Range	Mean		Mean	Range
Av. Length of Bout:	Patches	(1-3)	1.6	Bathsheba	3.4	(1-12)
	Martha	(1-6)	3.3	Max	4.0	(1-9)
	Whistler	(1-8)	4.3	Whistler	4.0	(1-11)
	Spike	(1-15)	6.3	Sue	3.8	(1-11)
				Isabella	5.4*	(1-13)

* Isabella entered cold 9/27/79
Other animals entered cold 11/29/79

H: in hibernation

S: semi-hibernation (slight movement)

A: active and alert

Fig. 2

EFFECT OF AGE (WEIGHT) ON HEART RATE OF NEW BORN HAMSTERS

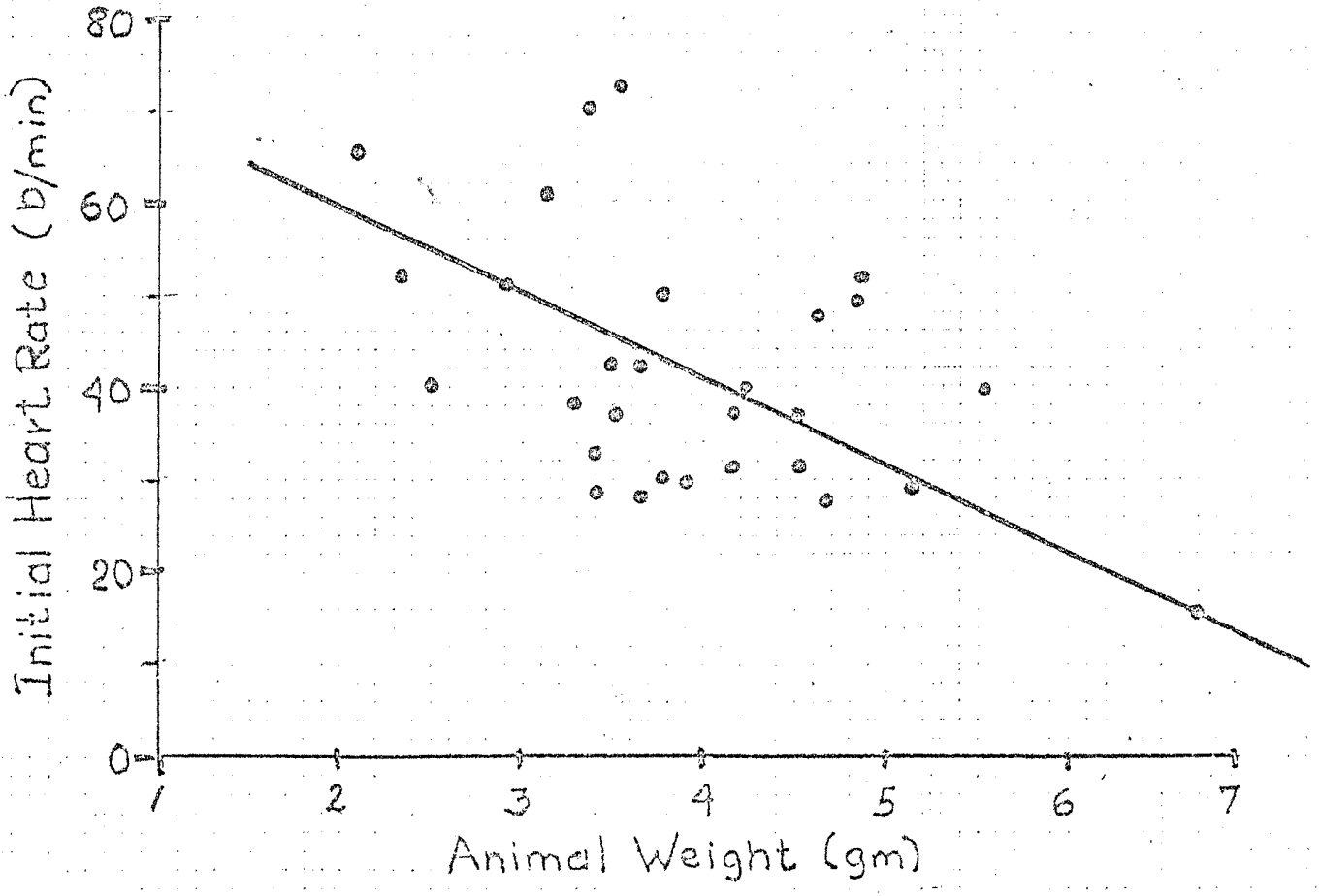


Fig. 3A

"CONTROL" HEART RATES OF
NEW BORN HAMSTERS
PERFUSED WITH RINGERS SOLUTION

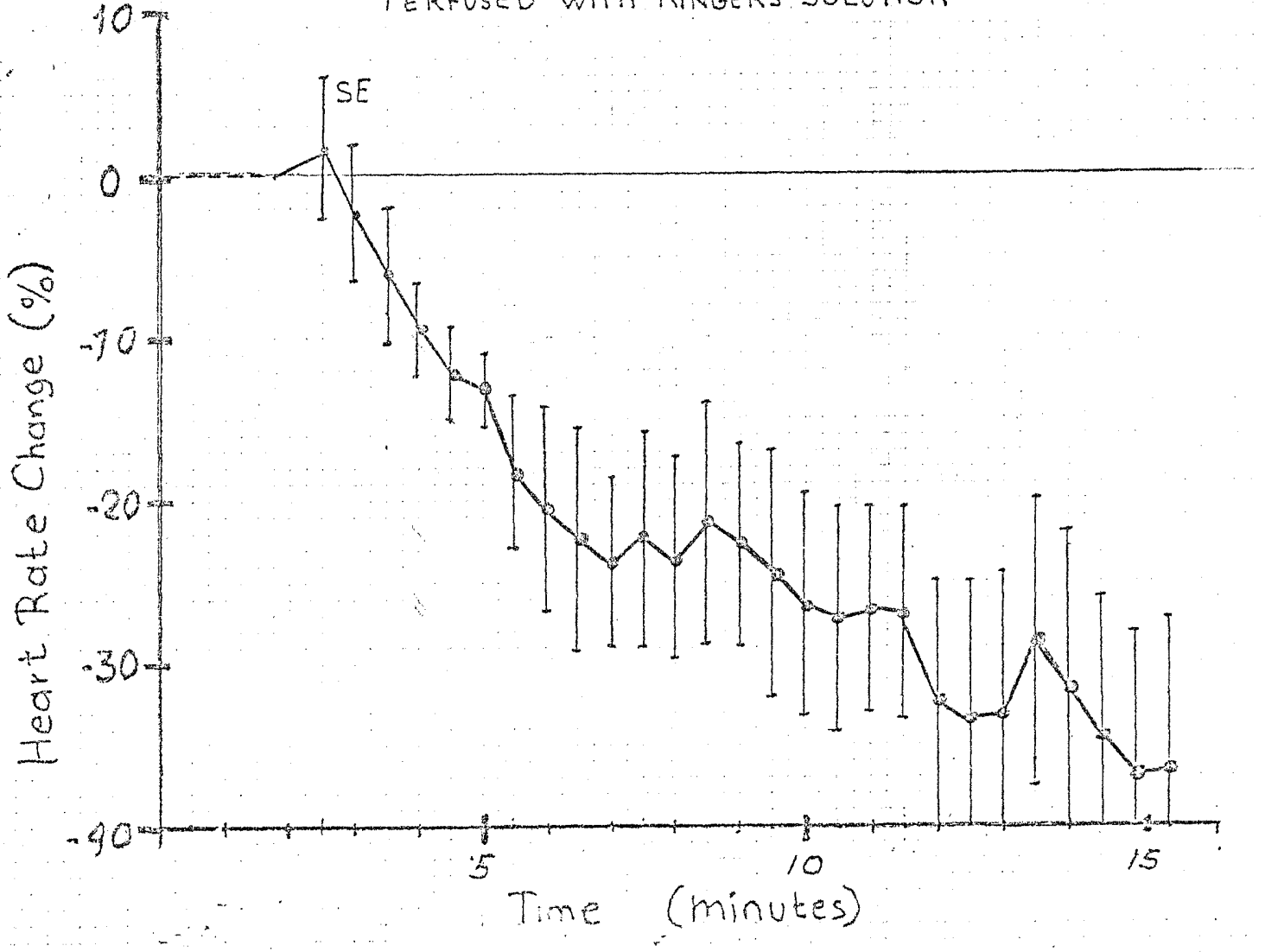
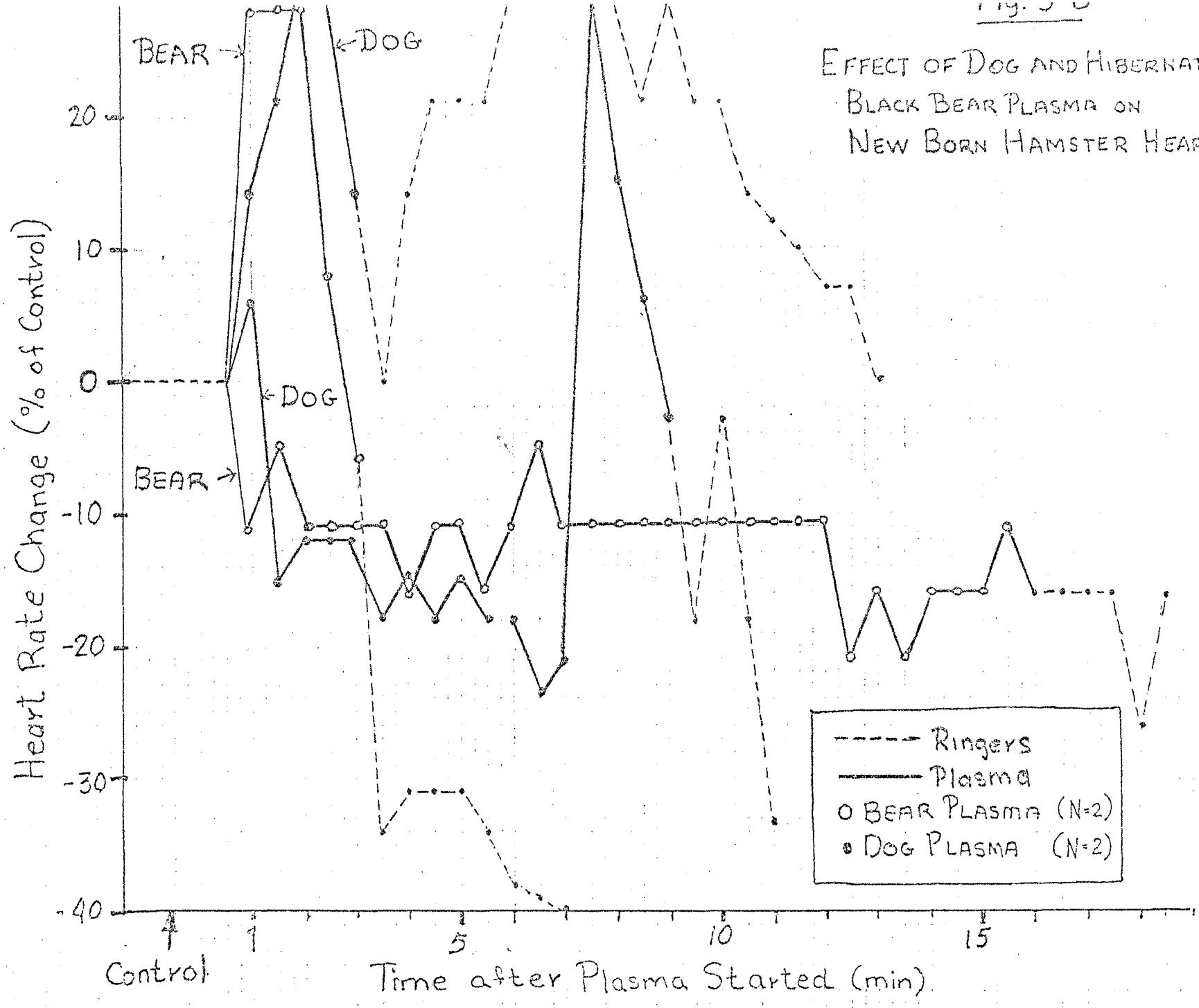


Fig. 3 b

EFFECT OF DOG AND HIBERNATING BLACK BEAR PLASMA ON NEW BORN HAMSTER HEARTS



APPENDIX A.

Procedure used by Dr. Oeltgen for separating and isolating hibernation-inducing trigger.

THE OELTGEN TECHNIQUE

Plasma from hibernating woodchucks was desalted utili a hollow fiber device having a molecular exclusion limit o 5,000. Such a device completely removes small molecules si as the catecholamines and unbound steroid hormones and vari ions such as calcium and magnesium, all of which have at va times been implicated for their ability to induce hibernati This thermolabile preparation was then further fractionated 4°C by isoelectric focusing (IEF), isotachophoresis and by passage through an affinity chromatography column. The latt procedure specifically separates albumin from the remainder the plasma fractions. The IEF fractionation in a pH gradien extending from 3.5-10.0 indicated that the HIT activity resi with the albumin fraction of the plasma. This still hetero-genous preparation having a pH range of 4.5 to 5.2 induced hibernation within 2-6 days when injected at a concentration of 3mg/ml in 8 out of 10 summer-active ground squirrels. All other plasma fractions were inactive at similar concentration This albumin fraction has been further separated by IEF in a narrower pH gradient extending from 4.0 to 6.0 and by isotac phoresis. These resolved fractions will be assayed for HIT activity in ground squirrels during midsummer as will the purified albumin fraction from the affinity chromatography column.

APPENDIX B.

Reprint of review article on hibernation trigger written during the period of this contract.

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MINIREVIEW

HUMORAL INDUCTION OF MAMMALIAN HIBERNATION

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Abstract—1. The terminology and physiology of hibernation are briefly reviewed.
2. Entrance into hibernation and possible blood-borne "trigger" substances are discussed.
3. Recent studies on "antabalone" and "hibernation trigger" are reviewed.
4. The role of naturally occurring substances capable of inducing hypothermia is discussed and related to the study of hibernation.

INTRODUCTION

Although the phenomenon of natural mammalian hibernation has been extensively investigated during the past century, the actual causes for the onset of this depressed metabolic and physiological state remain unknown. Through the efforts of many investigators it has been shown that the entrance into hibernation is under precise physiological control resulting in the dramatic lowering of heart rate, respiratory rate and oxygen consumption, followed by a decline in body temperature. In this state the hibernating animal conserves energy and is able to live this way for up to 9 months at temperatures just above freezing, depending on the species. Nonhibernators are unable to survive these dormant conditions for very long. But why does a mammal capable of hibernation that is not hibernating also die in the state of hypothermia, despite the fact that it can survive longer and at lower ambient temperatures than a nonhibernator? It has been suggested that the normal "physiological thermostat" is reset during hibernation, allowing the animal to be cooled in a controlled manner; but how this "resetting" is accomplished is little known (Lyman, 1961); possibly the dual neurochemical theory of Myers & Sharpe (1968) and Myers & Yaksh (1972) may prove to be the explanation.

There are three theories describing the causes and control of hibernation. These are: (a) hibernation is indicative of poor thermoregulation and is a primitive state in which the animal physiologically resembles the poikilothermic lower vertebrates—unable to resist the drop in temperature the animal becomes cold and so hibernates; (b) hibernation is triggered by a change in the levels of some substance or substances normally present in the mammalian body; and (c) the central nervous system is the site of the "physiological thermostat" and is the basic controlling mechanism (Lyman & Chatfield, 1955). The first theory has largely been rejected, for reasons discussed later. Evidence has been accumulating for the past 10 years that seems to indicate that either one or both of the latter two theories may be correct. In a series of exciting experiments, Dawe & Spurrier (1969, 1972, 1974b), Dawe *et al.* (1970) and Spurrier *et al.* (1976) have succeeded in isolating a substance called "hibernation trigger" from the bloods of hibernating 13-lined ground squirrels and woodchucks that acts interspecifically and will induce summer hibernation in these species. Recently, Swan *et al.* (1977) have prepared an extract called "antabalone" from the brains of hibernating 13-lined ground squirrels that decreases the metabolism and body temperature of rats for as long as 30 hr. Additional substances that are possibly related to the induction of hibernation or to inducing hypothermia include bombesin and related peptides isolated from the skin of various anuran species (Brown *et al.*, 1976, 1977a,b), opiates and endorphins (Bloom *et al.*, 1976; Tseng *et al.*, 1977), sleep peptides (Espamer & Melchiorri, 1975; Pappenheimer *et al.*, 1975; Schoenenberger *et al.*, 1977; Schoenenberger & Monnier, 1977; Uchizono *et al.*, 1975) and as yet unidentified transmitter factors released from the brain during thermoregulation (Myers & Sharpe, 1968), to name just a few of the suspected substances.

What is the relationship, if any, between hibernation trigger and antabalone? Do bombesin, opiates and any of the other related substances discussed above play a role in the induction of hibernation? These questions shall be examined here in the light of the research results to date.

Before discussing the literature dealing with the many substances suspected of being the "trigger" or stimulus for hibernation it will be helpful to review the physiology of hibernation. The next few paragraphs will also serve to distinguish hibernation from sleep, hypothermia and aestivation.

TERMINOLOGY OF HIBERNATION

Aristotle was the first to recognize that animals and birds "concealed themselves" (Mrosovsky, 1971) to avoid harsh environmental conditions either by escape in space (migration) or by escape in time (hibernation). Hibernation is a torpid state where torpor is defined as partial or total loss of sensibility or the power of motion (Kayser, 1965) associated with the onset, progression and maintenance of a markedly diminished metabolic rate and a profound state of

central nervous system depression resembling coma (Swan *et al.*, 1969). Thus, hibernators in torpor exhibit a general depression of their metabolism such that there is a profound decrease in heart rate, oxygen consumption, respiratory rate and core body temperature, as well as a loss of locomotor activity and decreased response to external stimuli. Aestivation is qualitatively the same as hibernation with the exception that the former occurs in response to a hostile environment that is hot and/or dry at body temperatures greater than 20°C while hibernation occurs in response to cold. Swan *et al.* (1969) in their work with the African lungfish, *Protopterus aethiopicus* consider the two phenomena to be different manifestations of a single basic process they describe as torpidation. There are indications that a torporous state can be induced autopharmacologically and out of season (Dawe, 1973). The phenomenon of hibernation can be subdivided into two types, poikilothermic and homeothermic. Poikilothermic hibernation is characteristic of the lower animals (invertebrates, lungfish, amphibians, reptiles) which at low air temperatures take on the temperature of their surroundings. They are forced into a cold torpor and cannot rewarm themselves until their environmental temperature increases. In homeothermic hibernators, mammals or birds which normally maintain their body temperatures near 37°C assume a state of greatly reduced core temperature while retaining the ability to spontaneously rewarm themselves back to their normal homeothermic temperatures without the absorption of heat from the environment (Folk, 1974).

HIBERNATION AND HYPOTHERMIA

Only hibernating mammals in dormancy can thermoregulate at near-freezing temperatures. Non-hibernating mammals in hypothermia behave like the poikilothermic lower vertebrates as will non-hibernating mammalian hibernators under the same conditions, although they can survive for longer periods at temperatures below 20°C that would be fatal to their non-hibernating relatives. Hibernation is a controlled and finely regulated condition, whereas artificial hypothermia results in a breakdown of temperature regulation causing a weakening or collapse of other homeostatic mechanisms (Lyman, 1961). Arousal of a mammal from artificial hypothermia requires the reintroduction of external heat but the arousal from natural hibernation depends upon some intrinsic heat-generating mechanisms for which the hibernator does not require external heating (Dawe & Spurrier, 1974a). It is interesting that hibernating mammals are poikilothermic at ambient temperatures between +5 to +20°C, but if the temperature goes below this range, then homeostatic thermoregulation occurs, which may or may not arouse the animal (Kayser, 1965).

HIBERNATION AND SLEEP

Is hibernation entered from a state of sleep or is it an extension of sleep? This difficult question has plagued hibernation researchers for many years. Sleep and hibernation can be said to share at least one similar function and that is the conservation of energy reserves due to a lowered metabolic rate, thus decreasing energy expenditure for the animal (Walker *et al.*, 1977). Animals in hibernation usually maintain their normal sleeping positions (Lyman, 1963).

Sleep spindles characteristic of Stage II sleep (slow wave sleep or SWS) appear during the entry into hibernation in EEG recordings. The occurrence of rapid eye movement (REM) sleep varies, depending on the species being studied (Mrosovsky, 1971). During SWS there is a slight drop in body temperature, a slowing of the heart rate, a decrease in oxygen consumption and greatly reduced muscular activity. The decrease in body temperature appears to be regulated in that it is readjusted downward during SWS (Dement, 1966), as it is during hibernation. Existing evidence supports the view that sleep and hibernation are physiologically homologous, although it is not conclusive. Recent results by Walker *et al.* (1977), using EEG activity and electromyographic recording (EMG) of muscle potentials as criteria in two species of ground squirrels, indicate that these species enter hibernation predominantly through sleep. In contrast to the findings of other workers, they found that below brain temperatures of 27°C there is little or no REM sleep as determined by EEG activity and that below 25°C it is primarily SWS, although it is difficult to interpret the recordings at these low temperatures.

It is clear that the hypothalamus contains centers for the control of both sleep and wakefulness and for temperature regulation; whether or not these centers are associated is not known. Neurosecretory cells in the supraoptic nucleus appear to increase in size as hibernation progresses (Suomalainen, 1960), but the significance of this neurosecretion and its relation to sleep is not presently understood.

In summary, the present evidence indicates that the entrance into hibernation occurs through slow wave sleep, at least in the two species of ground squirrels studied by Heller's group.

PHYSIOLOGY OF HIBERNATION

Mammalian hibernation occurs primarily within three orders: Chiroptera (bats), Insectivora (hedgehogs), and Rodentia (ground squirrels, woodchucks, hamsters), though there is evidence that some members of the marsupial and primate orders hibernate (Hoffman, 1964). Only in certain species within the first three orders cited does hibernation occur. The "winter lethargy of carnivores" such as bears and the hibernation of bats are special cases (Folk, 1974) and will not be considered here. The remainder of this discussion will be confined to members of the rodent order, with particular emphasis placed upon the 13-lined ground squirrel (*Citellus tridecemlineatus*) and the woodchuck (*Marmota monax*).

A common misconception about hibernation in these small animals is that it is a continuous state and the animal, upon entering this state in the fall, does not awaken until spring. Actually, hibernation occurs in cycles or bouts in which the animal periodically awakens or arouses, eats stored food if available, voids and returns to a state of metabolic torpor within 24 hr (Lyman, 1963). For example, the hibernation cycles of the woodchuck and the 13-lined ground

squirrel can be described as consisting of four phases: activity, induction, hibernation and arousal. The following description of the phases of hibernation is from Dawe & Spurrier (1972): (1) activity is the condition of an animal from spring to fall wherein it does not naturally hibernate; (2) induction represents the change which occurs (generally within a few hours) from the active or arousal state to the hibernation state; (3) hibernation is the condition of an animal seen periodically from fall to spring in which body temperature is lowered to within a degree or two of the environment (as low as 0 °C), respiration and heart are slowed profoundly, the animal is balled up, hair is erect; bouts of hibernation (each several days or weeks long) recur throughout the fall and winter season; and (4) arousal is the condition of an animal in which it looks like an active animal in all respects but is between two bouts of hibernation. It may last for a few hours or a few days and is usually followed by another bout of hibernation.

The cyclical manifestations of hibernation suggest that hibernators, like many other animals, keep their behavior and physiology in time with the changing environment in one of three ways: (1) a direct response to changes in the environment, usually photoperiodic; (2) an endogenous "clock" programmed to respond at a specific time regardless of any external stimuli; and (3) combined responses to the first two listed (Spurrier & Dawe, 1977). In accordance with this, Hoffman (1964) has listed three types of hibernators: (1) "permissive" hibernators like the golden hamster (*Mesocricetus auratus*) store and use food prior to hibernation and during periods of arousal, and hibernation is optional even in the winter; (2) "obligate" hibernators become hypothermic or are forced to hibernate under conditions of food deprivation or cold temperatures [the pocket mouse (*Perognathus longimembris*) is representative of this group], and (3) the vast majority of hibernators are classified as "seasonal" in that each year they experience a rhythm of preparation for the coming of winter and a period of hibernation, followed by a season of breeding and activity, as in the 13-lined ground squirrel and the woodchuck. Pengelley coined the term "circannual rhythm" for this predictable cyclic behavior (Folk, 1974).

What are the physiological characteristics of a hibernator in torpor? It has already been mentioned that the animal shows a decreased heart rate, respiratory rate, oxygen consumption and a decline in body temperature. The heart rate undergoes a decline of 90–95%, eg. 100 to around 5 beats/min. The respiratory rate and O_2 consumption drop in a similar manner. All three parameters are subject to fluctuation during a bout of hibernation (Folk, 1974). Core body temperature is decreased such that in most hibernators it is maintained at only 1 or 2°C above ambient temperatures. The respiratory quotient is very close to 0.7, which indicates that the small amount of energy required for maintenance is derived from the oxidation of fat which has been stored in the animal's tissues before hibernation. There seems to be a general involution of all endocrine glands during this time; thus, they all present a nonsecretory appearance (Benedict & Lee, 1938; Folk, 1974; Kayser, 1961, 1965; Mrosovsky, 1971). Metabolism and heat production may decline to 1/30–1/100 of the normal basal values for awake animals. Large oscillations in these values may precede entry into hibernation. Hoffman (1964) states that "by a complex interplay of muscular activity, shivering and vasodilation, particularly on the dorsal skin surface which is exposed to the cold, heat loss and heat production are coordinated so that the rate of temperature decline and the critical levels are always under rather precise control".

That these changes occur on the tissue level has been shown by Spurrier & Dawe (1977) for the isolated hibernating heart. The ability of the hibernator's cardiovascular and nervous systems to function at temperatures just above 0°C is striking. Nerve conduction in hypothermic nonhibernators ceases at around 9°C or above, whereas the nerves of hibernators will conduct down to 3°C. It has been shown that certain biochemical adjustments or a process of acclimatization take place in the central nervous system of the prepared hibernator (Hoffman, 1964).

ENTRANCE INTO HIBERNATION

The entry into hibernation may be characterized by a series of "test drops" where the brain temperature, as measured in the classic experiments of Strumwasser (1959), does not decline all at once, but in a series of steps occurring over several days for the California ground squirrel (*Citellus beecheyi*). Entry may happen in just a few hours, as in the case of the woodchuck. Simultaneous measurements of heart rate, O_2 consumption and body temperature performed by Lyman (1958) show that in the woodchuck entering into hibernation, heart rate drops first, followed by O_2 consumption and then temperature declines. Respiratory rate is the first to drop in some ground squirrels (Landau & Dawe, 1958).

Climatic factors thought to be involved in the induction of hibernation include a low ambient temperature, shortening of the photoperiod, season (fall or winter), and the "confined air" present in the animal's burrow (Kayser, 1961).

Possible trigger substances

Many different blood-borne substances have been implicated in the induction of hibernation; these may be referred to as "triggers" and include adrenal cortical hormones, insulin, an extract from brown fat (the "hibernating gland"), electrolytes (Mg^{2+}, Ca^{2+}, K^+), brain extracts, "antabolone" and "hibernation trigger". The adrenal cortex involutes during hibernation (although the zona glomerulosa may be active) while the medulla does not and the latter almost certainly plays a role in the awakening process. The exocrine pancreas involutes but the endocrine pancreas does not; the role of insulin in induction remains unclear (Kayser, 1965; Mrosovsky, 1971). Brown fat, also known as the "hibernating gland," has been shown to contain an arousal substance, but there is no evidence that it produces a hibernation-inducing substance (Dawe & Spurrier, 1974a). Of the serum electrolytes magnesium has received the most study and is present in higher levels during hibernation than in hypothermia (Folk, 1974). Of the possible factors listed above, only "hibernation trigger" and "antaba-

lone" (brain extracts) shall receive further consideration in this paper.

CURRENT THEORIES OF HIBERNATION INDUCTION

Although Swan (1963) first proposed that the lungfish's brown fat might be the source of an antimetabolic hormone, it is the brain that produces "antabalone". The extract from aestivating lungfish brains produced lethargy, a 35% drop in metabolic rate and a fall of 5°C in body temperature for at least 6 hr (Swan *et al.*, 1968, 1969), while the control rats showed no such effects. Nearly 10 years later, an antimetabolic extract was obtained from the brains of hibernating 13-lined ground squirrels by Swan's group (1977) that in rats induced almost identical effects as the lungfish extract. O_2 consumption decreased to 65% of control values within 30 min, the effect lasting from 90 min to 30 hr. The average decrease in body temperature was 5.25°C. This extract, dubbed "antabalone", can be cryogenically stored without losing its potency for several months. Similar results obtained from brain extracts of members of two different vertebrate classes support the concept that aestivation and hibernation are qualitatively the same and suggest that the same molecule, probably a small molecular weight polypeptide, is involved in both responses. Apparently, Kroll in 1952 (Swan *et al.*, 1969) has described an extract from the brains of hibernating animals which induced a state of slumber lasting 2–40 days, and resembling hypothermic sleep when injected into dogs or cats. Swan's extracts have not exhibited this degree of potency, but there are probably differences in the methods of preparation and experimentation involved. It is clear from the data that antabalone suppresses both metabolic rate and body temperature when given to nonhibernators. How antabalone affects hibernators has not yet been tested.

A blood-borne substance that actually "triggers" hibernation in 13-lined ground squirrels and woodchucks in the summer and is found only in the blood of hibernating animals has been described in a series of elegant experiments by Dawe & Spurrier (1968, 1969, 1971, 1972, 1974a,b, 1975), Dawe *et al.* (1970) and Spurrier *et al.* (1976). Their major findings can be summarized as follows:

(1) The "trigger" for hibernation is present in whole blood, washed cells and serum of 13-lined ground squirrels and woodchucks only in the hibernating state.

(2) It is not present in the blood of active or aroused animals or in blood of nonhibernators under hypothermic conditions.

(3) Trigger acts interspecifically between these squirrels and woodchucks with no immune responses.

(4) Hibernation trigger activity can be cryogenically preserved for up to 6 months.

(5) The trigger is effective as a serum or when it is dialysed; the non-dialysable residue is not effective as a trigger and evidence indicates that it may contain an antitrigger, as does the blood of active and aroused animals.

(6) The titer of trigger increases with the length of a bout of hibernation; trigger from a donor in a long bout of hibernation will induce hibernation more rapidly than from a donor in a short bout.

(7) Trigger is not effective when injected into cold-adapted animals.

(8) Female animals are more effective recipients than males whereas both are equal donors.

(9) The trigger will manifest its effect in winter whether a recipient animal is placed in a cold-dark or a warm window-lit room. (Sometimes hibernators will enter a state of torpor at a room temperature of around 25°C and will maintain their body temperature at 26°C. This aestivation-like state can be induced by hibernation trigger.)

(10) Hibernation induced artifically by a trigger in summer will not end the following winter; these animals continue to hibernate, sporadically, until death.

(11) Woodchuck hibernation serum is more potent than 13-lined ground squirrel serum.

Dawe & Spurrier (1972, 1974a) have advanced an interesting theory to explain their results. There are two factors of different molecular size involved: the dialysable small molecule (SM) that is the "trigger" and the non-dialysable larger molecule (LM) that is the "antitrigger". The binding of SM to LM forms a complex molecule (CM) which inactivities the trigger. This circannual "trigger-antitrigger" theory is discussed in terms of relative amounts of SM and LM present at different times of the year. Both SM and LM are absent in the young-of-the-year and "trigger" may decrease in concentration with age, explaining the weakening capability for hibernation as the animal gets older, although this is unclear. [For more details see Pivorun (1977).]

Unfortunately, the chemical nature of hibernation trigger is still unknown. Oeltgen *et al.* (1978a,b) are currently working on this problem and have determined that the trigger molecule is bound to or closely associated with albumin and that its physiological role may be dependent upon changing albumin concentrations. Earlier microscopic examination of the bloods taken from a 13-lined ground squirrel in the summer and from one hibernating in the winter revealed that small (1 μM) particles adhere to the red blood cells in the winter and are found in the plasma. Larger (10–30 μM) particles are seen in the plasma in the spring and summer and in nonhibernating 13-lined ground squirrels in the winter. These 1 and 10–30 μM particles might represent aggregates of SM and LM, respectively. The same (presumably) 1 μM particles (SM aggregates?) were found in high concentrations in vessels in the brain (Dawe & Spurrier, 1968), which suggests that there may be some connection between "antabalone" derived from the brain and hibernation trigger, if these particles turn out to be the same molecule(s). Confirmation of this must await specific chemical characterization of the two molecules.

OTHER SUBSTANCES RELATED TO HYPOTHERMIA/HIBERNATION?

Now let us consider the relationship of some other interesting substances, which have been the subjects of intensive study in recent years, to the induction of hibernation and/or production of hypothermia. Recall that hibernation differs from hypothermia in

that it is a controlled state where the animal can thermoregulate, while hypothermia is not. Nevertheless, substances that produce hypothermia in nonhibernators may prove to be natural induction substances in hibernators. Compounds to be discussed include bombesin and related peptides, opiates and endorphins, the sleep peptides, and neurotransmitter factors released from the brain during thermoregulation. Structure–activity relationships will certainly be of interest in this discussion.

Bombesin, xenopsin, physalaemin and litorin are vasoactive peptides that have been isolated from the skin of several anuran species that are related structurally to either neurotensin (NT) or substance P (SP). Neurotensin, isolated from the bovine hypothalamus, when given intracisternally but not intravenously, lowers the basal body temperature of mice by 4°C. SP shares several of the other functions of NT such as the lowering of blood pressure, but has not been found to lower body temperature (Brown *et al.*, 1976), while the other related peptides have less activity. Because both bombesin, resembling SP, and NT are active in this assay and yet have different structures, it has been proposed that they act at different receptor sites (Brown *et al.*, 1977a). Bombesin-like immunoreactivity has recently been found in the gastrointenstinal tracts of frogs, dogs, and even in humans (Espamer & Melchiorri, 1975; Walsh & Holmquist, 1976) and more recently, in the mammalian hypothalamus by Villarreal *et al.* (1978). Like somatostatin, vasoactive intestinal peptide, gastrin, NT and SP, (found in both gut and brain and presumably of neural crest origin) (Pearse, 1976), bombesin is also found in the central nervous system and may have some neurotropic role. The anuran skin glands that produce bombesin are of neural crest origin (Brown *et al.*, 1977a) and this lends credence to this idea. Although metabolic measurements have not been reported, it is worthy of notice that bombesin depresses core body temperature in the same way that antabalone does. It is possible that the structure of antabalone, once it is ferreted out, may be found to be similar to bombesin or one of the other peptides derived from anuran skin; perhaps bombesin *is* antabalone. Note, however, that Villarreal *et al.* (1978) have found that mammalian bombesin is chemically and immunologically different from frog skin bombesin.

Recently, it has been demonstrated that the hypothermic effects of bombesin in cold-exposed rats can be blocked by the administration of naloxone, an opiate antagonist (Brown *et al.* 1977b). This suggests that there is an opiate-dependent step involved in the mechanism of action of bombesin. Opiates alone will lower core body temperature in rats by as much as 4°C. β-endorphin is the the most potent opiate in this respect, and like the other effects of opiates and endorphins, this is naloxone-reversible (Bloom *et al.*, 1976). Tseng *et al.* (1977) were able to show that tolerance develops to the hypothermic effects of β-endorphin or morphine sulfate. Perhaps β-endorphin or some other endogenous opiate mediates the action of bombesin and related peptides in the induction of hypothermia. There is no apparent structural similarity between the groups of compounds.

Because of the seeming connection between sleep and hibernation, we shall now consider the sleep peptides as candidates for the induction of hibernation. At least three different research groups have purified preparations that induce sleep in experimental animals. These are from the brains of sleep-deprived rats (Uchizono *et al.*, 1975), sleep-deprived goats (Factor S) (Fencl *et al.*, 1971; Pappenheimer *et al.*, 1975), and from rabbits receiving constant stimulation of the intralaminal thalamic area (Factor Delta or delta sleep-inducing peptide, DSIP) (Schoenenberger *et al.*, 1977; Schoenenberger & Monnier, 1977). Only the structure of DSIP is known; it is a nonapeptide and does not resemble any of the members of the bombesin family or any of the members of the β-lipotrophin family. All of the sleep peptides have been found to reduce locomotor activity in rats, but no assessment of their effects on body temperature or metabolic rates have been performed as yet. All are dialysable, small molecular weight compounds, which suggests some similarity to hibernation trigger. It does not seem too far-fetched to suppose that an overdose of sleep peptide could induce a hibernation-like state.

Finally, Myers & Sharpe (1968) and Myers & Yaksh (1972) showed that neurotransmitter substances are direcly involved in thermoregulation. Perfusate collected from the anterior hypothalamus of a cooled donor monkey induced fever in a recipient while perfusate from a heated donor monkey induced hypothermia in the recipient. This supports the dual neurochemical theory of thermoregulation where intracisternal injection of serotonin is associated with hyperthermia and catecholamines with hypothermia. These neurotransmitters may change the ratio of Ca^{2+} to Na^{+}, which has also been shown to be involved in temperature regulation (Myers & Yaksh, 1972). A neurotropic peptide such as neurotensin or bombesin (or antabalone?) could easily be involved at the anterior hypothalamic level in the induction of hypothermia. It has already been stated that an opiate dependent step may exist in the mechanism of action of bombesin; perhaps a catecholamine is also involved.

SUMMARY AND CONCLUSIONS

The terminology and physiology of hibernation were presented, followed by a discussion of the entrance into hibernation and the current theories of hibernation induction. A blood-borne hibernation "trigger" and a brain extract called "antabalone" seem to be the best contenders at present for hibernation-inducing factors. The group of substances consisting of bombesin and related peptides, opiates and endorphins, sleep peptides and neurotransmitters may or may not be involved with the hypothermic response that is part of the hibernation induction process. They play a role in the thermoregulatory response or in the control of the level of an animal's activity, but the connection to hibernation is unclear. Obviously, much more experimental work in the area of hypothermia and hibernation induction is required.

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APPENDIX C.

Manuscript concerning polar bear blood.

CHARACTERIZATION OF THE PLASMA LIPIDS AND LIPOPROTEINS OF THE POLAR BEAR

Because elevated plasma lipid concentrations appear to predispose to the premature development of atherosclerosis, there is a great deal of interest concerning the composition and structure of the macromolecules in which lipids are transported in the circulatory system. These macromolecular complexes, the serum lipoproteins, are composed primarily of a heterogenous mixture of proteins, triglycerides, cholesterol, cholesteryl esters, phospholipids, and carbohydrates in the form of glycoproteins. Because of the ready availability of human blood, the human lipoproteins have been extensively characterized ( ). Recently, increased emphasis has been placed on obtaining similar data in a wide variety of animals. Such information should provide a more comprehensive understanding of the lipid transport system in mammals. Moreover, it may indicate certain lipoprotein properties that are found only in species susceptible to atherosclerosis, thereby providing important clues as to the relationship between lipoprotein structure and the development of atherosclerosis. The present study of polar bears ~~living in~~ their native habitat contributes additional and previously unavailable information to the comparative aspects of lipoprotein structure in animals.

TABLE 1

Lipid Composition of Polar Bear Blood Serum[1]

Bear	Day	Phospholipid (mg/dl)	Triglyceride (mg/dl)	Cholesterol		
				Free (mg/dl)	Ester (mg/dl)	Free/total
M	1	387	180	105	165	0.39
M	5	344	166	98	148	0.40
F	1	405	218	130	211	0.38
F	5	405	233	131	204	0.37

[1]Blood samples were obtained from a fasting male (M) and female (F) polar bear at five day intervals.

TABLE 2

Fatty Acyl Composition of Serum Lipids

Fatty acid	Composition %[1] Phospholipids	Triglycerides	Cholesteryl esters
Individual acids			
<16:0[2]	0.6 ± .1	1.2 ± .9	1.4 ± 0.1
16:0	11.6 ± .3	6.4 ± .4	7.8 ± 0.2
18:0	37.7 ± 0.8	4.7 ± .6	1.6 ± 0.1
16:1	5.5 ± 0.4	10.9 ± 1.6	14.6 ± 0.2
18:1	27.2 ± .1	33.8 ± 2.8	25.7 ± 0.9
18:2	2.5 ± .2	3.1 ± .4	5.0 ± 0.4
18:3	3.4 ± .6	4.6 ± 1.0	0.9 ± 0.2
20:4	3.4 ± .2	2.3 ± 0.1	11.4 ± 0.7
20:5	4.4 ± .1	12.7 ± .9	29.3 ± 1.4
22:5	0.8 ± .4	3.6 ± 0.4	0.4 ± 0.3
22:6	0.4 ± .3	13.7 ± 3.2	0.8 ± 0.1
Others	2.5 ± 0.4	3.1 ± 0.6	1.0 ± 0.2
Fatty acid classes			
Saturated	49.9	12.3	10.8
Monoenoic	32.7	44.7	40.3
Polyenoic	14.9	40.0	47.8

[1]Each values is the mean ± SE of 4 samples

[2]Total fatty acids containing less than 16 carbon atoms

TABLE 3

Lipid Composition of Plasma and Isolated Lipoprotein Fractions

Serum Fraction	Phospholipids (mg/dl)	Triglycerides (mg/dl)	Cholesterol	
			Total (mg/dl)	Esterified (%)
Intact Plasma	385 ± 14	199 ± 15	298 ± 24	61 ± 0.4
VLDL	7 ± 1	30 ± 7	9 ± 2	0
LDL	70 ± 5	167 ± 13	121 ± 19	51 ± 3
HDL	308 ± 19	5 ± 1	170 ± 6	78 ± 1

Figure 1

Column Chromatography of Isolated Polar Bear Serum Lipoproteins.

After preparative ultracentrifugation, the lipoprotein fractions were further purified using agarose column chromatography. A 2.5 cm I.D. x 40 cm column packed with Biogel™ 50 M from Bio-Rad Industries was employed. 2.5 ml of the lipoprotein sample was supplied to the column and 0.154 M NaCl containing 0.1 mg/ml of EDTA was used to develop the column. The flow rate was 50 cm/hr, and 3.9 ml fractions (80 drops) were collected. Absorbance was measured at 280 nm. The solid line represents the elution profile of the isolated LDL fraction and the dashed line represents the profile of the isolated HDL fraction.

Figure 2

SDS-Disc Gel Electrophoresis of Isolated Polar Bear Apo HDL

Apo HDL samples were prepared by adding SDS to a final concentration of 1%. Fifty µl of polar bear apo HDL in 10% glycerol were layered in 12.5% gels. 2-Mercaptoethanol was not added in the electrophoresis system in gel (a), but it was present in gel (b). Electrophoresis was carried out at 8 mAmps per gel for 8 hr using a 0.01 M phosphate buffer containing 0.1% SDS, pH 7, according to the method of Weber et al. ( ). Staining with Coomasie blue and destaining was done according to the method of Fairbanks et al. ( ).