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AUTONOMIC CONTROL OF CIRCULATION DURING THE HIBERNATING CYCLE IN GROUND SQUIRRELS

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It has been assumed for several decades that the autonomic nervous system plays an important role in the phenomenon of hibernation in mammals, but definitive evidence has not been available. Until recently investigations of the entrance into hibernation and the hibernating state were perforce descriptive, for any physical disturbance of the animal usually started the process of arousal. During arousal various physiological manipulations could be carried out and the effects noted, so that this phase of the hibernating cycle is moderately well documented (Lyman & Chatfield, 1955). From these data it has been postulated that the sympathetic nervous system is importantly involved in the process of arousal. On the other hand, it has been suggested that entrance into hibernation is brought about by parasympathetic influence (Strumwasser, 1960) or lack of sympathetic influence (Britton, 1928), but evidence to substantiate this is scanty.

Techniques of chronic cannulation of major blood vessels (Still & Whitcomb, 1956) have opened a new approach to the study of hibernation. By means of an indwelling cannula, drugs of known pharmacological effect can be introduced into the blood stream in minute amounts and the effects noted, with the assurance that the changes observed are due to the drug alone. With suitable devices, blood pressure, pulse pressure, heart rate, body temperature and the electrocardiogram can be obtained at will. Using these techniques, we have attempted a partial dissection of the role of the autonomic nervous system throughout the hibernating cycle.

METHODS

The thirteen-lined ground squirrel (*Citellus tridecemlineatus*) was the principal experimental animal used in this study, but comparative observations were made on the golden-mantled ground squirrel (*C. lateralis*). A total of 116 animals were cannulated, of which 57 *C. tridecemlineatus* and 2 *C. lateralis* supplied useful data in a total of 323 definitive experiments. All experiments were repeated at least three times, on at least two animals.

Only animals which had been hibernating were cannulated. The technique for cannulation has been given elsewhere (Lyman & O'Brien, 1960). A thin polyethylene tube (PE 10, outer diam. 0·61 mm, 0·28 mm bore), sealed at one end, was implanted in the abdominal aorta with the open end facing up-stream about 1 cm above the renal arteries and the sealed end protruding from the skin of the mid back. In a few animals the external jugular vein was also cannulated so that a drug could be introduced on the venous side. After cannulation the animal was returned to the hibernaculum and usually re-entered the hibernating state. It was then fitted with an iron-constantan thermocouple which was sewed subcutaneously in the region of the heart, with the wires making their exit beside the aortic tube on the mid dorsum. The aortic tube was spliced to a length of PE 10 tubing by means of a section of 27-gauge hypodermic needle. This tube and the two thermocouple wires were protected by a light helical spring which was attached to the back of the animal. In some animals three silver-wire electrodes were sewed into the skin of the back for e.c.g. and e.m.g. recording.

The animal was placed in a round battery jar (23 cm diameter) with ample bedding, food and water. The helical spring was led through a screen covering the top of the jar, and suspended at the end with an elastic band and a fishing swivel so that the animal could move freely. Usually five or six cannulated ground squirrels were kept in this manner at $5 \pm 2°$ C in a cooled, insulated box.

Body temperature was monitored on a Speedomax thermo-electric recorder, type G, with an accuracy of $\pm 0{\cdot}25°$ C (Leeds and Northrup, Philadelphia, Pennsylvania). The indwelling thermocouples were made of 36-gauge wires, each protected throughout its length with PE 10 tubing. Corrosion of the iron wire was reduced by filling this tube with a flexible epoxy resin. For acute colonic temperatures a thermocouple was inserted into the rectum to a depth of 2–3 cm.

When measurements were made, the protective helical spring was steadied with a clamp, the thermocouple wires were attached to the potentiometer, and another length of PE 10 tube was spliced to the aortic tube, making a total length of about 70 cm. The recording system from the tip of the catheter was flat ($\pm 5\%$) to a sinusoidal wave pressure disturbance of 320–340 c/min. Careful comparisons with anaesthetized animals showed that neither the length of the tube nor its temperature made appreciable differences in the recorded blood pressure within the conditions of the experiments (Lyman & O'Brien, 1960). The tube led to an infusion apparatus consisting of a 1 ml. syringe fitted with a motor-driven screw drive. By using this apparatus, the animal could be infused steadily at the rate of 0·2 ml/hr. If the drive was manually turned the perfusion rate could be as high as 0·1 ml. in 4 sec, with an accuracy of 0·001 ml. A Statham pressure transducer, model P 23 D (Statham Instruments Inc., Los Angeles, California) was attached to a T in the copper tubing which led from the infusion apparatus to the attachment of the polyethylene aortic splice. The transducer was amplified with a Grass low-level DC pre-amplifier, model 5 P1 A, and a Polygraph DC driver amplifier, model 5 (Grass Instrument Co., Quincy, Massachusetts), and the excursions recorded on an ink-writing oscillograph. A mercury manometer, set at the level of the animal and attached to another T in the tubing, was used for calibration.

In testing the effect of a drug a record was made of the pulse pressure until a typical pattern was established. A measured amount of the drug was then introduced into the polyethylene splice with a Krogh–Keyes pipette. The fluid containing the drug was isolated on each side from the neutral infusion fluid by a short (5 mm) column of air. Tests with a coloured liquid and heparin saline showed that the air columns prevented mixing of the two liquids much more effectively than oils or other substances. The short air columns did not affect the contour of the pulse pressure, as was shown by comparisons before and after introduction of the bubble.

Before infusion of the drug the same amount of neutral fluid (7·5 mg heparin/100 ml. physiological saline) was infused into the animal as a control. The drug could then be

introduced either as a whole, or by small increments with the screw drive of the infusion apparatus. In the latter case the first dose of the drug could not be exact because the precise volume of the whole tube was unknown and because some mixing of the two fluids always occurred, but once there was a pure column of the drug at the open end of the cannula, as little as 0·001 ml. of the drug-containing fluid could be introduced with reproducible physiological results. The results of infusing the various drugs indicated that the course of the liquid was almost invariably in the direction of the blood flow, i.e. caudally. Very occasionally, however, when the heart rate was slow and the drug was infused quickly, the rapidity of the result indicated that the drug had been forced up-stream into the coronary circulation.

Doses throughout this paper are reported in mg/kg. They are calculated by assuming 150 g as the average weight of the ground squirrel. Because weighing disturbed the active animals and aroused the hibernators they were not weighed routinely, unless an animal was obviously over- or underweight.

In determining the drug dosage for animals in the various stages of hibernation, the substance was first introduced via the aortic tube into animals which were not hibernating but in the 'active' state and the dose established for the required result. (We use the word 'active' to describe a potential hibernator when not in any phase of actual hibernation: the 'active' animal could be actually asleep or immobile.) With exceptions which will be noted, doses of the same magnitude were used for animals in deep hibernation.

As has been emphasized previously (Lyman & O'Brien, 1960), infusion of any drug, even isotonic saline, occasionally causes cardio-acceleration, a rise in blood pressure and sometimes arousal from hibernation. Therefore, a drug was not considered to be cardio-acceleratory unless it invariably speeded the heart. When infusion of a drug produced no observable effect on a hibernating animal there was no absolute assurance that the drug had the same pharmacological potency as it had on an active animal. The effectiveness of atropine (atropine sulphate; Mallinckrodt) in hibernation was tested by briefly choking the animal at the beginning of the arousal process. Asphyxia at this time produces a vagally-induced bradycardia (Chatfield & Lyman, 1950), and abolition of the bradycardia indicated heavy atropinization. In studies on the para-sympathetic system no vagotomized animals were used, because the operation divides the recurrent laryngeal nerve and respiration becomes blocked by mucus.

In order to stimulate the vagus nerve of the hibernating animal, electrodes were fashioned of fine stainless-steel wire, each in a polyethylene tube (0·23 mm bore). Near each tip one side of the tube was cut away, exposing the wire. The right vagus nerve of previously intubated animals was dissected free and the exposed part of each wire was wrapped around the nerve, with the intact portion of the polyethylene serving as a shield. The electrodes were stitched in place, with a polyethylene spreader separating them by about 0·5 cm. The two insulated wires were led to the mid dorsum and passed through the protective helical spring. Necrosis of the nerve always occurred and the useful duration of such a preparation was never more than 1 week. A Grass stimulator (Grass Instrument Co., Quincy, Massachusetts) was used to produce biphasic shocks of 20 msec duration at a frequency of 25/sec.

By the techniques outlined above studies were made on active animals, and on animals entering hibernation, in deep hibernation, and arousing from hibernation. Of the four phases, the second was most difficult to study, because the onset of hibernation is unpredictable. Furthermore, the animals are extremely sensitive at this time. Any stimulation is apt to change this phase into the very different phase of arousal, so that investigations on entering hibernation have been necessarily limited.

RESULTS

Entering hibernation

When the ground squirrel enters hibernation a fall in heart rate and blood pressure always precedes the decline in body temperature. The slowing of the rate is accomplished by a lengthening of the period between the even beats and by actual skipped beats which appear at fairly regular intervals (Lyman & O'Brien, 1960). As the temperature declines the even beats become slower, followed by longer periods of asystole (Fig. 1*A*).

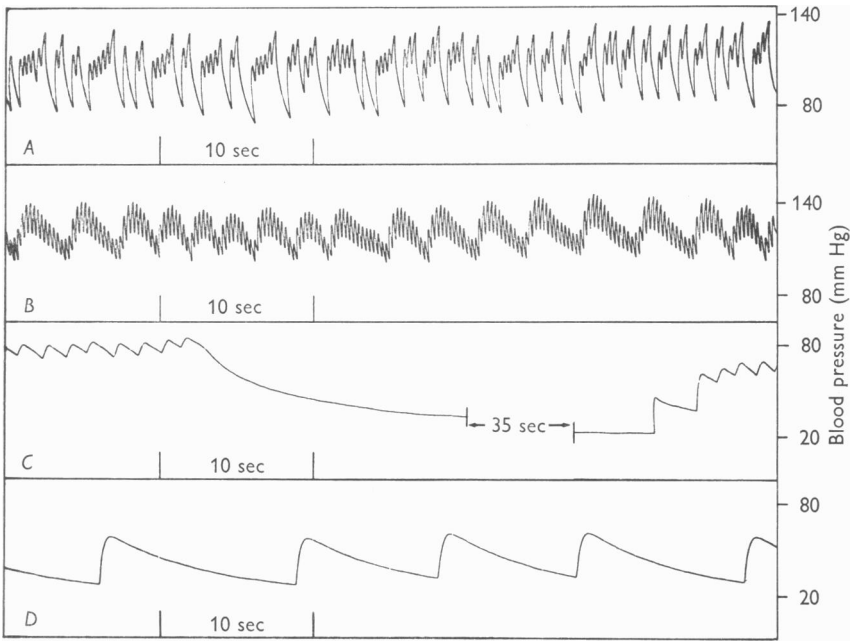

Fig. 1. *A*. Typical pulse pressure pattern of thirteen-lined ground squirrel entering hibernation, showing skipped beats. Thoracic temperature 29·5° C. *B*. Same animal 10 min after treatment with atropine, which abolishes skipped beats. Thoracic temperature 29·0° C. *C*. Long asystole in atropinized animal entering hibernation. Asystole lasted 58 sec in this case. Thoracic temperature 14·5° C. *D*. Atropinized animal (18·5 mg/kg) with pulse pressure pattern which is typical of untreated ground squirrel in deep hibernation. Thoracic temperature 10·6° C.

This 'interrupted saw-tooth' type of pulse pressure may result in a very low average heart rate as the animal approaches the deeply hibernating state, in spite of the intermittently rapid heart. Heart rate plotted against either time or body temperature (Fig. 2) results in a smooth curve. If only the periods of even heart rate are counted, the temperature–rate relation is much less exact (Fig. 2).

Parasympathetic blockade. Although the heavily atropinized animal will enter hibernation, only one complete record was obtained, for the drug accelerates the heart and the animal becomes more alert. Once body temperature has declined a few degrees it usually continues to drop to 6–7° C after treatment with atropine or methantheline bromide 1·3 mg/kg (Banthine bromide; G. D. Searle and Co.), in spite of the abolition of skipped beats, a temporary increase in the frequency of even beats and

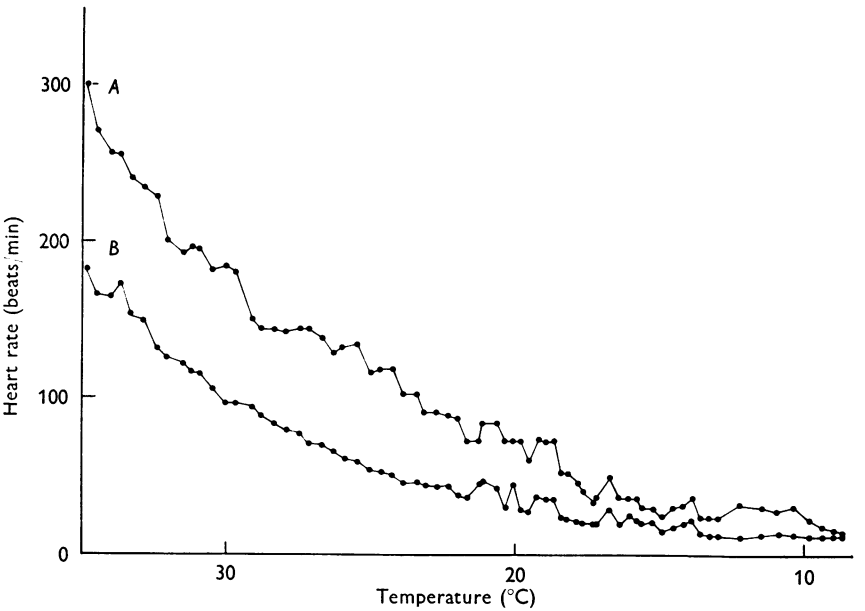

Fig. 2. Temperature–heart-rate curves of a normal ground squirrel entering hibernation. Heart rate registered for a 1 min period at 5 min intervals. *A*. Rate calculated by counting only the evenly occurring beats. *B*. Actual heart rate; note fewer irregularities in curve *B*.

an elevation of the blood pressure (Fig. 1*B*). Occasionally an animal rewarmed at once after infusion of either drug. In one case the body temperature continued to decline slowly from 23 to 13° C, and fluctuated from 10·6 to 14° C for 15 hr before rewarming.

Although atropine abolishes the evenly spaced skipped beats, extended periods of asystole, lasting as long as 86 sec at a body temperature of 15·6° C, may occur (Fig. 1*C*). As the asystole continues the respiratory rate increases and a bodily 'flinch' often occurs before the heart resumes beating. At this point the rate is relatively rapid, but decreases again as the respiratory rate slows. As the animals grow colder the flinches cease, the periods of asystole become more frequent, and the periods of tachy-

cardia shorter, until the typical pulse pressure of deep hibernation is reached (Fig. 1*D*). In contrast to the skipped beats of the normal animal entering hibernation, these asystoles cannot be under parasympathetic influence, for they occur with doses of atropine as high as 24 mg/kg, while less than one half this dose will abolish the reflex bradycardia caused by choking the arousing hibernator, and only 3 mg/kg will abolish vagal slowing caused by startling the active animal.

Atropinization (3·6–4·1 mg/kg) is particularly effective in the last stages of entering hibernation, when the heart rate is very slow, but the body temperature is still declining. Two animals with body temperatures of 10° C and heart rates of 6–10 beats/min increased their rates threefold, and another with the same heart rate and a temperature of 14° C increased the rate to 63 beats/min. In these and other cases the body temperature continued to decline.

Because of the presence of skipped beats, the heart rate of an untreated animal entering hibernation is slower than that of an atropinized animal at the same body temperature. However, if only the *even* rates are compared it is found that this rate is not necessarily slower in the untreated animal. In atropinized animals, where prolonged asystoles occur, the average heart rate approaches that of the control but the temperature–rate curve is very uneven.

Parasympathetic stimulation. The study of the effect of parasympathomimetic drugs on the heart rates of *C. tridecemlineatus* and *C. lateralis* while entering hibernation or in the hibernating state was complicated by the fact that infusion of either acetylcholine (acetylcholine chloride; Merck) or methacholine (Mecholyl chloride; Merck) in a wide variety of doses caused cardio-acceleration before either drug could circulate to the heart (see *In hibernation*, p. 483). We had no opportunity to test the effect of either drug on an animal entering hibernation when fitted with a jugular cannula, nor were we able to stimulate the vagus electrically during this phase of the hibernating cycle.

Sympathetic blockade. The infusion of the sympatholytic agent β-TM10 ([2-(2,6-dimethylphenoxy) propyl]-trimethylammonium chloride hydrate; Smith, Kline & French) in doses as high as 13·8 mg/kg did not hasten the onset of hibernation in active ground squirrels which recently had been hibernating. Because the initial effect of this drug is a transient but marked cardio-acceleration, it could not be used to study the effect of sympathetic blockade during the process of entering hibernation, for infusion resulted in immediate arousal.

In hibernation

Even when only temperature and heart rate are considered, the transition from entering hibernation to the deeply hibernating state is not clear-cut. The heart slows to the rates found in deep hibernation while the body temperature is still several degrees above the environmental temperature, yet body temperature continues to decline for 4 hr or more thereafter (Lyman & O'Brien, 1960). Finally, the body temperature becomes stable 2–3° C above the environmental temperature.

It may be categorically stated that deep hibernation has been attained under these conditions, yet the interrupted saw-tooth type of pulse pressure, which is typical of entering hibernation, can persist for many hours at average rates which are as low as the relatively even rates of hibernation. Throughout the hibernating period, the heart rate varies from day to day and sometimes from hour to hour, with rates usually falling between 5 and 8 beats/min at heart temperatures of 3–8° C. However, rates as slow as 2 and higher than 10 beats/min were recorded in the normal hibernating ground squirrel.

Pace-maker. In order to test whether the variation in heart rate was due to an alteration in the inherent rhythmicity of the pace-maker, veratramine 0·98 mg/kg was infused into hibernating animals in an attempt to slow the heart (Krayer, 1949). Though the respirations became deeper there was no change in heart rate, and normal cardio-acceleration occurred when the animals were mechanically disturbed. As they warmed they underwent violent convulsions, indicating that the dose, at least during the waking process, was sufficient to affect the heart, for the amount of drug which produces convulsions is considerably higher than the amount which influences the heart (Krayer, 1949).

Parasympathetic system

Effect of atropine. The effect of parasympathetic blockade during deep hibernation contrasted with its effect during entrance into hibernation. In animals with heart rates of 2–12 beats/min and steady heart temperatures between 3·5 and 8° C, atropine (4·1–41·0 mg/kg) often did not increase the rate at all, though if there were unevenly occurring periods of asystole they were abolished, causing a more even rate. Blood pressure remained unchanged. Extremely slow heart rates (2–4/min) showed no more tendency to increase after atropinization than faster rates. Methantheline bromide (1·5–6·4 mg/kg) usually, but not always, caused transient cardio-acceleration lasting about 1 hr in deeply hibernating animals, the rate then returning to below 10 beats/min.

Effect of acetylcholine. The initial cardio-acceleratory action of acetylcholine and methacholine complicated the study of these drugs on hibernating animals. However, in a large series of experiments on over 45 *C. tridecemlineatus* and *C. lateralis*, infusion of acetylcholine or methacholine never slowed the heart rate of the undisturbed hibernating animal in spite of a wide variety of volumes, infusion rates and doses (acetylcholine 0·013–8·2 mg/kg and methacholine 0·1–1·0 mg/kg). Pre-treatment with physostigmine 0·16 mg/kg (physostigmine sulphate; Merck) also failed to produce the typical para-sympathomimetic effect of acetylcholine. Some time after either acetylcholine or methacholine had accelerated the heart, infusion of the same drug caused a series of prolonged asystoles. These asystoles appeared 9–15 sec after the infusion, and must reflect the circulation time from the end of the catheter to the heart. Sometimes another asystole occurred 20 sec or more after the first, probably caused by the drug circulating to the heart a second time. On occasion slowing of the accelerated heart occurred almost immediately after infusion of the drug. In these cases it was assumed that some infusion fluid had been forced up-stream into the coronary arteries.

In animals which had been in hibernation for 1 day or more the change in threshold to the parasympathomimetic effect of acetylcholine or methacholine took place at least 15 min after the first infusion of the drug had caused cardio-acceleration, but often occurred more rapidly if the animal had just entered deep hibernation. This change took place even if the body temperature and faster rate remained static, indicating that time and an accelerated rate were important factors. Thus, in a typical case acetylcholine 6·2 mg/kg caused the heart rate of a hibernating *C. tridecemlineatus* to increase from 7 to 30 beats/min in 12 min. At this point infusion of the same dose had no effect. Seven minutes later, with the heart rate still 30, the identical dose caused transient slowing of the heart. Body temperature remained at 8·5° C throughout this time. When infusion of acetylcholine caused the ground squirrel to arouse from hibernation, the minimum dose necessary to produce asystoles could be determined at various periods. Once the typical parasympathomimetic effect was established, the threshold to the drug dropped within a few minutes to one tenth or less of the concentration needed originally to produce cardiac slowing, and the threshold was changed very little thereafter as the animal warmed. However, such a preparation does not truly test the threshold response of the heart with changing temperature, for the blood-borne drug must circulate to the heart, and the change in activity of cholinesterase during arousal from hibernation is unknown.

Effect of stimulating the vagus nerve. Because of the accumulation of cellular debris around the vagal electrodes, precise comparisons of voltage

thresholds were only valid within each experiment. Stimulation with as much as 140 V for 10–60 sec failed to produce slowing of the heart during deep hibernation. The stimulation produced a contraction of the neck muscles, and the heart rate increased as if the animal had been disturbed manually. Stimulation with the same voltage immediately after the heart rate had increased failed to cause slowing, but after about 1 hr a prolonged asystole could be produced, even when the body temperature had risen to only 9° C. As the animal warmed and the heart rate increased further, progressively lower voltages were needed to produce asystole, until an absolute threshold as low as 20 V was reached when arousal was about two thirds complete.

A similar result often occurs naturally if a deeply hibernating animal is lightly stimulated. The heart immediately accelerates, sometimes with no change in heart temperature, and remains at the new rate without advancing further toward arousal. After a period of at least half an hour prolonged asystoles begin to occur quite regularly. The asystoles become more frequent with time and the pulse pressure record assumes the typical interrupted saw-tooth appearance of the normal animal entering hibernation, with the heart responding to vagal influence. After some hours the pulse pressure returns to the typical pattern of deep hibernation.

Effect of asphyxia. A cruder, but effective, test of the ability of the parasympathetic system to slow the heart in hibernation depended on the reflex slowing of the heart during asphyxia. If deeply hibernating ground squirrels were manually choked (using a glove in order not to warm the neck region) the heart was not slowed. Choking elicited arousal, with cardio-acceleration and warming. A series of prolonged asystoles could be produced by choking, 15 min or more after the initial stimulus. As with acetylcholine, there was no precise correlation with body temperature. One heart failed to slow at 10·6° C, while another slowed at 8° C.

Cardio-acceleration; acetylcholine-methacholine

The anomalous cardio-acceleratory effect of acetylcholine and methacholine provided a unique means of studying the factors controlling the heart rate in hibernation, and this was explored in detail. In some cases infusion of either drug caused a drop in blood pressure and peripheral resistance, and the cardio-acceleration which followed after several minutes could be reasonably considered to be compensatory (Lyman & O'Brien, 1960). In a more common type of response with a wide range of doses of acetylcholine (0·01–82·0 mg/kg), cardio-acceleration usually occurred within two heart beats after infusion of the drug, with an immediate rise in blood pressure. A similar result occurred with methacholine (0·1–1·0 mg/kg) but the cardio-acceleration was delayed at least 24 sec after infusion.

During this time a slight decline in diastolic pressure sometimes, but not invariably, occurred. With either drug the increased rate always lasted many minutes and was often followed by arousal from hibernation, particularly when large doses were employed.

By starting with low doses the minimum amount of parasympathomimetic drug necessary to produce an immediate increase in heart rate in the deeply hibernating animal was determined. This minimum dose was as low as 0·01 mg acetylcholine/kg or 0·1 mg methacholine/kg, but often varied from day to day in the same animal with the same body temperature and comparable heart rate. This variation may reflect the route of the drug through the circulatory system, and this route may be modified by changes in resistance in parts of the vascular bed from time to time. There was little difference between a dose which caused a speeding of the heart with subsequent return to the slower rate of deep hibernation, and a dose which initiated complete arousal, so that many animals were awakened from hibernation unintentionally. Pretreatment with physostigmine 0·16 mg/kg potentiated the reaction, reducing the minimum effective dose of acetylcholine by about one half and causing a longer period of cardio-acceleration. Atropine 6·15 mg/kg blocked vagal slowing in the active animal for several hours, but only partially blocked the cardio-accelerating effect of acetylcholine in hibernating ground squirrels, for doubling the dose of acetylcholine overrode the effect of atropinization.

Ganglionic blockade was produced by infusing the hibernating *C. tridecemlineatus* or *C. lateralis* with hexamethonium chloride 64·1 mg/kg (Hexameton chloride; Burroughs Wellcome & Co.). This caused a drop in peripheral resistance, indicated by a marked decrease in diastolic run-off time (Lyman & O'Brien, 1960) and a drop in blood pressure with a transient compensatory increase in heart rate. When acetylcholine (0·08–0·62 mg/kg) was infused, the heart rate either remained unchanged or dropped to a lower rate after a time commensurate with the circulation time to the heart. The blood pressure dropped even lower, so that despite artificial respiration, heat and infusion of L-norepinephrine (noradrenaline) one animal failed to survive. Lower doses of hexamethonium caused a drop in blood pressure but failed to block the cardio-accelerating action of acetylcholine.

The use of β-TM10 as a sympatholytic agent was complicated because infusion with a variety of doses and rates caused a cardio-acceleration and rise in blood pressure, which often resulted in arousal from hibernation, particularly if the infusion rate was slow. In some animals treated with β-TM10 9·2 mg/kg the heart rate returned to its original level within 24 hr. Infusion of acetylcholine 6·2 mg/kg always caused cardio-acceleration in these cases. However, in three animals the cardio-acceleration

caused by the same dose of β-TM10 lasted only 1–3 hr and the rate then dropped to 4–12 beats/min, with a lower blood pressure and peripheral resistance. Infusion of acetylcholine then caused a further reduction in blood pressure, but no cardio-acceleration, and prolonged asystoles were produced when the drug reached the heart. This syndrome was as lethal as that produced by hexamethonium, though neither drug produced untoward effects with the same doses in active animals.

In active ground squirrels anaesthetized with pentobarbital sodium 80 mg/kg (Nembutal sodium; Abbott Laboratories) rapid aortic infusion of acetylcholine (1·0 mg/kg or more) resulted in typical parasympathomimetically induced asystoles 5–6 sec after infusion, often with a drop and subsequent recovery of blood pressure. The recovery of blood pressure was sometimes accompanied by cardio-acceleration, which could be abolished by β-TM10 9·2 mg/kg. Pretreatment with physostigmine 0·16 mg/kg reduced the amount of acetylcholine necessary to produce cardiac slowing by about one half. Unlike the case during hibernation, cardio-acceleration was never a primary result of acetylcholine infusion.

The first noticeable effect of acetylcholine (0·75–9·2 mg/kg) was a twitch of the hind legs occurring within 1·5 sec after infusion, followed by the slowing of the heart and pilo-erection of the tail, then by contraction of the bladder, increased peristalsis and a respiratory gasp. Atropine (9·2–24·6 mg/kg) abolished the cardiovascular and visceral effects produced by acetylcholine, but the nicotinic effects could not be completely blocked though three or four times as much acetylcholine was needed to produce them.

The nicotinic effects were examined in hibernating thirteen-lined ground squirrels previously fitted with three electrodes sewed to the skin of the back. Acetylcholine in doses as low as 0·02 mg/kg resulted in a long-lasting burst of muscle action potentials but no visible movement, followed at once by cardio-acceleration (Fig. 3*A*). An almost imperceptible respiratory-like dorsal flexing occurred 16 sec or more after infusion, and, as the faster heart rate continued, respiratory rate also increased. Identical bursts of muscle action potentials followed by cardio-acceleration and increased respiratory rate were induced by physically poking the animal with a stiff wire, and usually resulted in arousal from hibernation (Fig. 3*B*).

Methacholine 0·31 mg/kg gave rise to no muscle action potentials immediately after infusion into the hibernating animal but caused one or more respiratory-like movements 24–180 sec later, which could be seen grossly and appeared on e.c.g. as bursts of muscle action potentials. These were followed by cardio-acceleration.

The time of onset of the nicotinic effects of acetylcholine, in contrast to methacholine, coincided with the difference in the onset of cardio-

acceleration caused by these two drugs in the hibernating ground squirrel. This suggested that induced muscular activity might be associated with cardio-acceleration during hibernation, and the invariable appearance of muscle action potentials before cardio-acceleration, whether the animal was stimulated pharmacologically or physically, supported this concept.

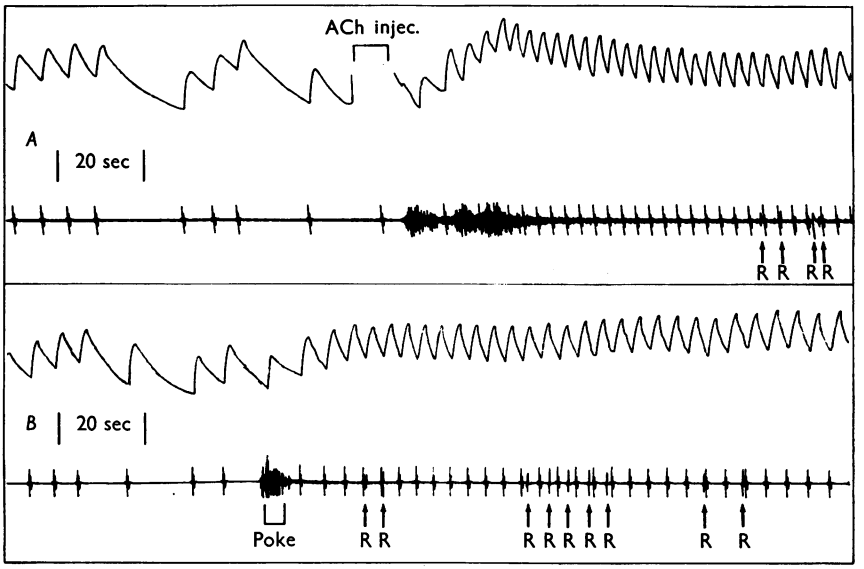

Fig. 3. *A*. Muscle action potentials and cardio-acceleration caused by infusion of acetylcholine (0·205 mg/kg). Note increase of respiration (R) after the stimulus. Thoracic temperature 9° C. Animal did not arouse from hibernation with this stimulus. *B*. Muscle action potentials and cardio-acceleration caused by poking same animal with a stiff wire 4 hr after *A*, with similar results. Thoracic temperature 8·6° C.

Therefore, hibernating ground squirrels were curarized (D-tubocurarine chloride pentahydrate (0·7–0·9 mg/kg); Abbott Laboratories) before treatment with acetylcholine. Unfortunately this caused a transient increase in heart rate, so that it was necessary to wait 30–55 min after infusion of the drug before the heart returned to its original slow rate. By that time the respiratory muscles were at least partially paralysed, and with the dose of curare 0·9 mg/kg the development of A–V dissociation indicated that the heart was anoxemic. Under these conditions infusion of acetylcholine or physical stimulation of the animal produced no muscle action potentials and no cardio-acceleration. With curare 0·7 mg/kg muscle action potentials could be reduced, but not abolished, when acetylcholine was infused. However, acetylcholine (0·05–6·2 mg/kg) never (6 cases) caused cardio-acceleration and never produced the typical respiratory-like move-

ment observed in the uncurarized animal, even though both these effects were produced by acetylcholine 0·05 mg/kg before curarization. With acetylcholine 6·2 mg/kg the heart slowed briefly 30 sec or more after infusion, evidently owing to the direct action of acetylcholine. In all cases warming and artificial respiration were necessary to rouse the animals.

Arousal from hibernation

As has been described previously (Lyman & O'Brien, 1960) an increase in heart rate and often a decrease in peripheral resistance are among the first changes observed when the hibernating animal starts the arousal process. Because of the decrease in peripheral resistance, the blood pressure does not necessarily increase at once, but eventually it rises as the heart beats faster. With the increase in heart rate and blood pressure the anterior part of the body warms, while the posterior remains cool. The blood pressure is at its peak value at about the time the thoracic temperature reaches 37° C. At this point the post-thoracic portion of the body warms quickly while the heart rate remains rapid and the blood pressure usually declines somewhat.

Infusion of various drugs confirmed the view (Lyman & Chatfield, 1955) that the differential rewarming was caused by vasoconstriction. During normal arousals animals were quickly infused with acetylcholine. Immediately after infusion, the thoracic temperature stopped its rapid rise and the rectal temperature began to increase. Typical asystoles sometimes occurred as the acetylcholine reached the heart. When the vasodilatory effect of the drug was dissipated, the rectal temperature ceased to rise and the heart again warmed rapidly. Repeated injections caused a rise and a plateau of thoracic and rectal temperatures, each a rough mirror-image of the other (Fig. 4).

The vasoconstrictor action of norepinephrine was tested during the last stages of arousal when the rectal temperature was rising rapidly and the thoracic temperature had reached 32–38° C. Rapid infusion of a very large dose (0·04 mg/kg) stopped the increase in rectal temperature for 2–3 min, but continuous infusion could not maintain this condition. If infusions were given serially several minutes apart, the rectal temperature could be made to rise in a stepwise manner (Fig. 5). Norepinephrine would not augment the increase in heart rate once arousal was under way nor did heavy atropinization increase the heart rate for any given temperature.

Hibernating ground squirrels infused with β-TM10 9·2 mg/kg aroused when mechanically stimulated 24 hr later. Under these circumstances the deep rectal temperature increased almost as rapidly as the thoracic temperature and remained no more than 4–5° C colder than the thoracic temperature during the whole arousal. With vaso-constriction absent the

warming process was greatly prolonged (Fig. 6). With this preparation animals were given a constant infusion of norepinephrine 0·007 mg/kg/min, to which were added rapid infusions of 0·008–0·045 mg/kg, so that in one case a ground squirrel received 0·13 mg/kg in 6 min. Even this large and continuing dose did not reimpose the differential rewarming typical of normal arousal, though the increase in rectal temperature was not quite

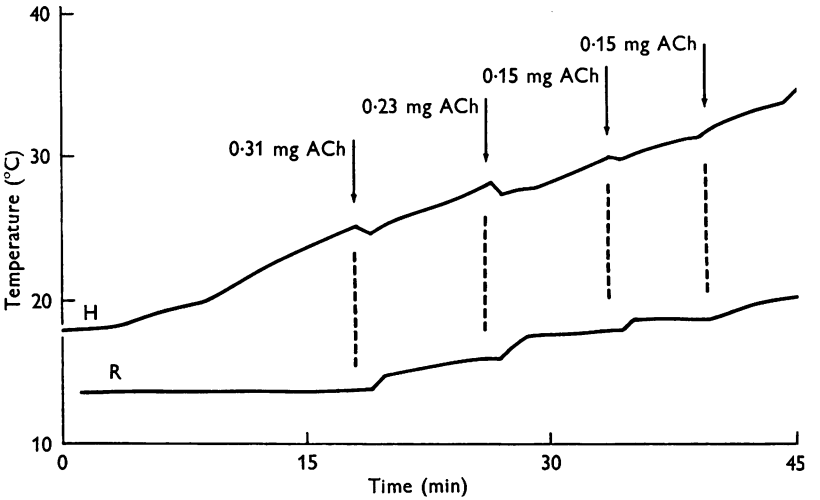

Fig. 4. Effect of acetylcholine on arousing hibernator while thoracic temperature is rising and rectal temperature is not. H = temperature in area of heart; R = rectal temperature.

as rapid during the period of infusion. Since β-TM10 does not block the effect of norepinephrine at the effector organ (McLean, Geus, Mohrbacher, Mattis & Ullyot, 1960), the experiment indicates that the differential vasoconstriction of arousal is not due to a lower threshold in the posterior part of the body.

DISCUSSION

Although the autonomic nervous system has been implicated theoretically in the phenomenon of hibernation, direct approaches to the problem are rare. Various drugs have been injected into active animals with the hope of producing the hibernating state, but to our knowledge all such attempts have been unsuccessful. Bilateral removal of the cervical sympathetic ganglia has been thought to hasten the onset and increase the duration of hibernation (Arbuzov, 1950), but the author does not suggest how this lesion produced the reported result. The role of the autonomic system in deep hibernation has also not been explored. We have emphasized previously that subcutaneous injections during hibernation

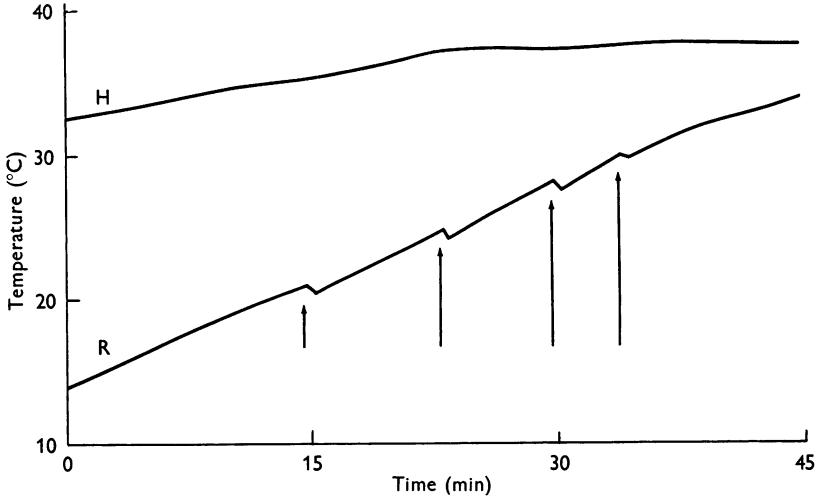

Fig. 5. Effect of norepinephrine (0·006 mg. at arrows) on animal in last stages of arousal when rectal temperature is rising rapidly. H = heart area; R = rectal temperature.

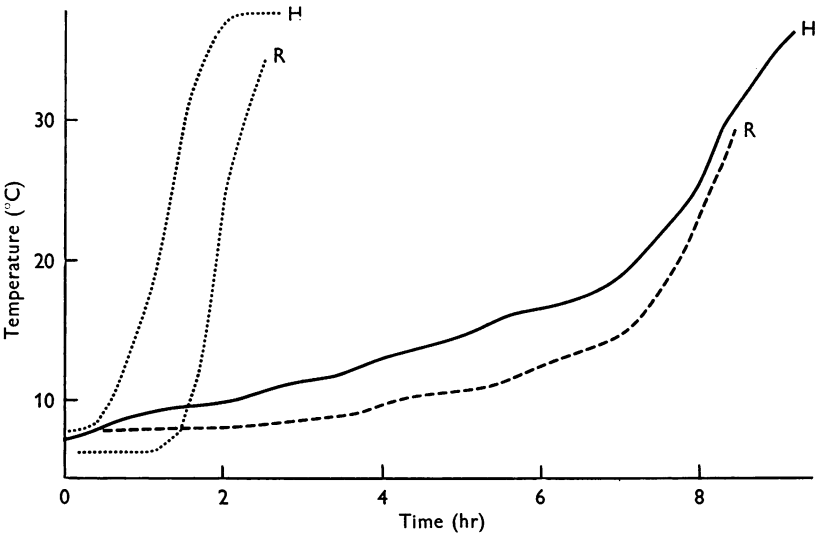

Fig. 6. Normal arousal from hibernation (dotted lines). Arousal after treatment with sympatholytic β-TM10 9·2 mg/kg (solid and interrupted lines). H = temperature in area of heart; R = rectal temperature.

cannot be sufficiently controlled to justify conclusions because response to physical stimulation is so variable. It is only during the acute process of arousal that experiments have been carried out which are sufficiently discrete to justify some conclusions (Lyman & Chatfield, 1955).

As far as entrance into hibernation is concerned, observations in the last decade have indicated that this is indeed a controlled process rather than a simple reversion to a primitive poikilothermy. The slowing of metabolic rate (Lyman, 1958), respiratory rate (Landau, 1956), and particularly heart rate (Lyman, 1958; Lyman & O'Brien, 1960) before any detectable decline in body temperature strongly suggest that these vital functions are being repressed by something more than changes in temperature alone.

The experiments reported here show that the normal skipped beats serve to regulate the decreasing heart rate as the animal enters hibernation. As the skipped beats are abolished by atropine or methantheline bromide, they must be mediated by the parasympathetic system. However, since the temperature continues to drop and the heart rate to decline after atropinization, the parasympathetic system is not essential for entering hibernation, and the decline in temperature may be the only cause for the slowing of the even heart rate in the atropinized animal.

Paradoxically, the prolonged asystoles which occur in some atropinized animals entering hibernation may be due to the absence of vagal influence. The asystoles first appear only after the heart has been beating at an even rate for some time, and this rate is faster than the over-all rate of an unatropinized animal at the same body temperature. During the asystole the lack of circulation is sufficiently severe to cause obvious respiratory distress before the heart starts to beat again. This strongly suggests that some sort of fatigue is involved in the cardiac arrest. Since the asystoles do not appear in the isolated hearts of ground squirrels as they are chilled (Lyman & Blinks, 1959), it is reasonable to assume that the heart of the atropinized ground squirrel is driven too fast for that temperature by the sympathetic system and ceases to beat when fatigue supervenes or sympathetic influence declines.

In atropinized animals, where asystoles occur, the periods of asystole can become more frequent as the atropinized animal approaches the deeply hibernating condition, until the heart rate is indistinguishable from that of the unatropinized animal in hibernation (Fig. 1*D*). If the fast heart in atropinized animals is caused by an overbalance of sympathetic activity, then the gradual decrease in the periods of cardio-acceleration may indicate a decline in this sympathetic tone. In the cases where the heart rate remains rapid, it may be postulated that the sympathetic tone does not decline enough to allow the heart to slow.

If this theory is correct, the sympathetic influence on the heart may

vary each time the animal enters hibernation, and parasympathetic activity may be essential in bringing the heart rate to a level typical of deep hibernation when sympathetic tone is high. This is emphasized by the fact that atropine causes immediate cardio-acceleration when infused into an animal with a heart rate typical of deep hibernation, but a body temperature that is still declining. Furthermore, the temperature–heart rate curves of woodchucks (Lyman, 1958) and ground squirrels are not the same during each entrance into hibernation, which may well be due to an interplay between sympathetic and parasympathetic activity.

The influence of the parasympathetic system on the heart rate once deep hibernation has been reached is minimal, if not completely absent, as is shown by the lack of effect of parasympathomimetics, parasympatholytics and electrical or reflex stimulation of the vagus. Furthermore, while immediate slowing of the heart occasionally took place when acetylcholine was forced up-stream in animals entering hibernation or during arousal, this never occurred during the deeply hibernating condition, in spite of the fact that the majority of experiments were performed on animals in that state. If the heart is accelerated and beats at a faster rate with the same temperature for some minutes, its threshold to acetylcholine decreases markedly. The actual rate of the heart at the time does not seem to be the factor which determines the sensitivity to parasympathetic influence, for the very slow hearts of animals treated with β-TM10, hexamethonium or curare are slowed by acetylcholine. In every case, however, the heart had been accelerated a short time previously.

A shift in the internal milieu or changes in some component of the heart itself, such as membrane potential, must cause this resensitization. Presumably the rise in threshold to parasympathetic influence takes place during the early part of deep hibernation, and may increase throughout the period of dormancy, though this has not been conclusively demonstrated. It is clear, however, that the heart of an animal which has just entered hibernation recovers its sensitivity to acetylcholine after a shorter period of acceleration than the heart of an animal which has been hibernating for a long time.

Whatever the cause, we have been unable to slow the heart of the ground squirrel in normal deep hibernation, though the rate is easily increased by a variety of drugs. If the parasympathetic system is without influence on the heart during this time and yet the heart beats slowly, it is logical to assume that sympathetic influence on the heart is also at a low ebb, but that any increase in sympathetic influence will produce a maximum effect.

Working with the California ground squirrel (*Citellus beecheyi*) Strumwasser (1959) found that the heart rate of this species was faster and more

uneven when plotted at 10 min intervals during deep hibernation than it was when the animal was entering the hibernating state. He suggested that similar changes in rate had been overlooked in *C. tridecemlineatus* and the European hedgehog (*Erinaceus*) by Dawe & Morrison (1955), and that these changes indicated an increase in sympathetic tone once the deeply hibernating condition had been reached. The present investigation confirms the work of the latter authors, for the slowest and most even rates occur during deep hibernation in *C. tridecemlineatus* and *C. lateralis*. On the other hand, the rate may increase if the animal is lightly touched or otherwise stimulated, and the faster rate may last for hours. Some thirteen-lined ground squirrels, particularly during the summer, are 'nervous' hibernators. Their heart rates tend to be fast in spite of the usual low body temperature, and the rate accelerates with even the slightest stimulation, such as infusion of isotonic saline. Several animals increased their heart rate every time the compressor of the cooling unit started, with the heart gradually slowing when the muffled noise and vibration ceased. Thus, there may be varying levels of sympathetic influence on the heart even in the same species at identical body temperatures, and it is reasonable to assume that there are species differences as well.

Alternative explanations for variations in heart rate during deep hibernation are not supported by our observations. If there are changes in the inherent rhythmicity of the pace-maker, they are not revealed by infusion of veratramine. Variations in the condition of the vascular bed might reflexly cause a change in rate, but we have found no absolute correlation between mean blood pressure and heart rate in normal hibernation. When the rate is above 10/min, the blood pressure tends to be higher than usual, indicating that the fast rate is increasing the blood pressure, rather than high blood pressure reducing the heart rate.

The long life at low temperatures which occurs only in hibernating animals may be due in part to the maintenance of a reasonably high blood pressure with a very low heart rate, for Popovic (1960) has shown a positive correlation between high blood pressure and prolonged survival time in chilled ground squirrels and rats. We have previously emphasized that the increase in peripheral resistance as the animal enters hibernation is an important factor in maintaining the blood pressure (Lyman & O'Brien, 1960). Infusion of acetylcholine, methacholine or β-TM10, ganglionic blockade or adrenergic blockade (Lyman & O'Brien, 1960) all may cause a reduction in peripheral resistance and blood pressure. The responses to these infused drugs indicate that the peripheral resistance is due in large measure to neurogenic vasoconstriction mediated by the sympathetic nervous system. The fact that drugs can produce such a striking effect

with no change in body temperature shows that physical factors such as changes in blood viscosity and elasticity of arteriolar walls at low temperatures cause only a part of the peripheral resistance.

The cardio-acceleration caused by infused acetylcholine or methacholine in the hibernating animal is worthy of attention because it may indicate the cause of the increase in heart rate which invariably occurs as soon as arousal begins in various species of hibernators (Lyman & Chatfield, 1950; Dawe & Morrison, 1955; Lyman, 1958; Lyman & O'Brien, 1960). Blocking of the acceleration by pre-treatment with hexamethonium or β-TM 10 indicates that the effect is produced via the sympathetic cardio-accelerator fibres, and the lethal effect of these drugs emphasizes the importance of vascular tone during hibernation and of cardio-acceleration during waking. Since methacholine lacks significant ganglionic effects except in very high doses (Goodman & Gilman, 1955), the drug-induced cardio-acceleration is not caused by direct ganglionic stimulation. The possibility of either drug producing a direct effect on the heart by release of endogenous catechol amines, or by some other means, is untenable because of the time factor. Circulation time from the end of the cannula to the heart is at least 9–15 sec, with a heart rate above 20 beats/min. The cardio-acceleration can occur 2–5 sec after injection of acetylcholine with the slower heart rate of deep hibernation. Our incidental observation that acetylcholine never increases the rate of isolated ground squirrel hearts at any temperature substantiates this conclusion. The effect could not be caused by direct stimulation of the central nervous system, for the time lapse would be even longer. Moreover, the passage of the acetylcholine molecule across the blood–brain barrier must be inhibited owing to its size.

The nicotinic effects produced by acetylcholine and methacholine are similar in hibernating and anaesthetized animals, for only acetylcholine produces a burst of muscle action potentials immediately after infusion whereas both drugs cause exaggerated respiratory movements at a time commensurate with the circulation of the drug to the anterior part of the body. None of these effects can be completely blocked by atropine. Both acetylcholine and methacholine produce reduction of heart rate in the anaesthetized animal, which can be readily blocked by atropine. In contrast, the invariable cardio-acceleration caused by acetylcholine in the hibernating animal can be only partially blocked by much heavier atropinization.

The time of onset of muscular activity after infusion of acetylcholine or methacholine in the hibernating animal coincides precisely with the onset of cardio-acceleration. The effect may be universal in hibernators, for the long-lasting burst of muscle action potentials produced by mechanically

stimulating the hibernating hamster is accompanied by cardio-acceleration (Lyman & Chatfield, 1950). Since curarization blocks cardio-acceleration caused by acetylcholine, the relation between muscular activity and cardio-acceleration seems to be established. Thus we would postulate a more complex reflex for cardio-acceleration in hibernation than the suggestion that increased thoracic movements mechanically stimulate the heart musculature (Dawe & Landau, 1960). In this regard the report that the skeletal muscle from animals in hibernation is much more sensitive to acetylcholine than muscle from active controls is of interest (Wachholder & von Ledebur, 1932).

The importance of the sympathetic and the inactivity of the parasympathetic component of the autonomic nervous system during arousal has been documented previously using drugs (Lyman & Chatfield, 1955) and other techniques (Dawe & Landau, 1960). The present paper shows that the heart can respond to parasympathetic influence, yet confirms with another species that its acceleration is not increased by parasympatholytics. Since the rate is not affected by sympathomimetics, the heart must be driven maximally by the sympathetic system, yet the adrenal medulla must play but a secondary role. The initial cardio-acceleration of arousal usually takes place too soon after stimulation to be caused by blood-borne adrenal catechol amines, though the delayed acceleration sometimes seen after infusion of acetylcholine might be caused in this way. Furthermore β-TM10 can block arousal, yet this drug does not influence the adrenal medulla (McLean *et al.* 1960).

The differential rewarming during arousal is clearly due to a more rapid circulation in the anterior part of the body (Johansen, 1961; Bullard & Funkhouser, 1962). Acetylcholine briefly abolishes the vasoconstriction of the posterior part during early arousal, and norepinephrine re-imposes vasoconstriction in the same area during the terminal phase of arousal. The vasoconstriction of natural arousal can be abolished by pharmacologically blocking the sympathetic nervous system, and it cannot be re-imposed by infusion of norepinephrine. Therefore the constriction appears to be caused by discrete action of sympathetic fibres rather than a lower threshold in the posterior to circulating norepinephrine.

We may therefore conclude that the parasympathetic system performs a regulatory, but not essential, function as the ground squirrel enters hibernation. If sympathetic tone is high, the parasympathetic system becomes more important in slowing the heart during entrance into hibernation, but throughout deep hibernation and arousal its effect is minimal and the animal with parasympathetic blockade shows no deficit. On the other hand, sympathetic activity must be reduced to permit the heart to slow as the animal enters hibernation, and the main function of the

sympathetic system during this period is to maintain sufficient vascular tone. Although the activity of the sympathetic system is muted in deep hibernation, it plays an essential part in the maintenance of circulatory homoeostasis, and if the system is blocked the animal dies of circulatory collapse. The sympathetic system remains on guard in the precarious hibernating state to be fired into action for its essential part in the complex co-ordinated process of arousal while the parasympathetic system remains in abeyance. It seems probable that the cardio-acceleration, which is an essential part of this process, is produced in evoked arousals when an external stimulus causes a burst of muscle action potentials which result in a reflex speeding of the heart.

SUMMARY

1. The control of circulation in undisturbed *Citellus tridecemlineatus* and *C. lateralis* has been studied during all phases of the hibernating cycle by infusing drugs into the blood stream through chronically implanted aortic cannulae and by monitoring pulse pressure.

2. The onset of hibernation is not hastened by infusion of a sympatholytic agent. Asystoles, which are typical of the animal entering hibernation, serve to maintain a more even temperature–heart rate curve as the animal enters hibernation. Parasympathetic blockade abolishes these asystoles, though the animal will still enter hibernation. Atypical prolonged asystoles sometimes occur in atropinized animals and are believed to reflect the cessation of sympathetic activity on the fatigued heart as the animal cools.

3. During hibernation veratramine does not alter the heart rate. Parasympathetic blockade often does not affect the rate and neither vagal stimulation nor parasympathomimetics slow the heart unless it has been first accelerated. This change in sensitivity is associated with continuance of a faster rate rather than change in temperature.

4. Acetylcholine and methacholine cause cardio-acceleration in deep hibernation, which is only partially blocked by atropine and is potentiated by eserine. Hexamethonium or β-TM10 causes a decline in peripheral resistance and blood pressure, and abolishes the cardio-acceleratory action of parasympathomimetics, indicating mediation via sympathetic cardio-acceleratory fibres. Cardio-acceleration, produced pharmacologically or by mechanical stimulation, is immediately preceded by muscle action potentials. Curarization abolishes the potentials and the cardio-acceleration, thus suggesting a reflex.

5. During hibernation peripheral resistance and blood pressure may be reduced by parasympathomimetics, or by ganglionic or sympathetic

blockade. Thus circulatory homoeostasis is normally maintained by sympathetic activity.

6. During arousal the normal vasoconstriction of the posterior part of the body can be abolished by pre-treatment with a sympatholytic agent and this cannot be re-established by norepinephrine, indicating that the differential vasoconstriction of arousal is due to discrete sympathetic nervous activity rather than to a local difference in threshold to circulating sympathomimetics. When the anterior part is warming, acetylcholine causes brief vasodilation of the posterior, and norepinephrine briefly reimposes vasoconstriction of the posterior part later in arousal when that area is warming rapidly. Atropinization has no effect on heart rate or circulation during arousal and norepinephrine will not further accelerate the rapidly beating heart.

7. It is concluded that the parasympathetic system has a regulatory, but not essential effect on the heart rate as the animal enters hibernation, and has minimal effect during hibernation and arousal. Decline in sympathetic influence contributes to cardiac slowing during entrance into hibernation but sympathetically controlled vasoconstriction is necessary for life in hibernation, and sympathetic mediation is essential for the precisely timed vascular changes involved in arousal.

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