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INVESTIGATION OF PEROGNATHUS AS AN EXPERIMENTAL ORGANISM
FOR RESEARCH IN SPACE BIOLOGY
(Contract NASw-812)

A SUMMARY OF PROGRESS
3 January 1967 through 30 September 1967

R. G. Lindberg
PRINCIPAL INVESTIGATOR

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HYPOXIA INDUCED HYPOTHERMIA AND HEMOGLOBIN OXYGEN
AFFINITY IN THE GENUS PEROGNATHUS

Page Hayden

The classification of mammals into the broad categories of "hibernator" and "non-hibernator" is based upon the individual species ability to spontaneously re-warm from body temperatures that are lower than "normal." These abnormal body temperatures may be evoked by depriving the animal of oxygen so that the heat producing processes are curtailed while essential metabolic reactions continue. The lowering of body temperature, in turn, establishes new requirements and equilibrium of all processes involved in a particular thermal realm. This continued cycling of decreased oxygen concentration and resultant cooling of the animal can be pursued to nearly any level of depressed body temperatures desired. Upon restoration of normal oxygen concentration, the "hibernator" (or facultative homeotherm) possesses specialized physiological mechanisms, i.e., brown fat, selective vasoconstriction, basic nerve differences, etc., by which it can rewarm to normal body temperature. While the obligate homeotherm may live for a time at reduced body temperature, it will eventually die. Not only is life limited at reduced temperature; there is also a temporal limit at which the animal may remain at the reduced temperature and be revived successfully (1).

Within the genus Perognathus (pocket mice) are species that are known to express daily periods of moderate hypothermia or even deep hypothermia (dependent upon prevailing ambient temperature) in relatively unstressed laboratory conditions (food, bedding and nest chamber). One of these species (P. longimembris) expresses an annual winter period of inactivity in its natural state. P. californicus has been documented to express a laboratory induced moderate hypothermia in direct response to decreased food supply. This torpor in P. californicus is interesting in that the lower limit of survival is 15°C (2). The species apparently cannot re-warm if its body temperature falls below the critical value.

The report of a lower temperature limit for survival in torpid P. californicus aroused interest in determining thermal limits for other species within the genus. It also raised the related question of possible correlations between oxygen-binding characteristics of the blood and the various degrees of facultative homeothermy observed in Perognathus sp. Of equal interest, perhaps, was the possibility of relating one or more of these characteristics to the high resistance to ionizing radiation exhibited by several members of this genus, thereby providing a clue to the mechanism of radiation resistance in Perognathus.

METHODS AND MATERIALS

The methods of producing, regulating and monitoring the response of confined, but not restrained, animals has been presented in detail (3). Briefly, animals with abdominally implanted temperature sensing telemeters are exposed to various hypoxic mixtures of oxygen and nitrogen in a cold chamber with animal temperature response and oxygen concentrations being recorded.

The evaluation of the oxygen binding capacity of the blood of the rodents required the development of micro-techniques to handle the small quantity of blood that was available from the smaller species (i.e., P. longimembris weighing 10 gm). The alternative was to pool blood samples for the macro-techniques available. This, in turn, would require the exsanguination, and consequent sacrifice, of relatively large numbers of the smaller species (which were in limited numbers).

The technique used was built around the Natelson Microgasometer (Scientific Industries, Queens Village, N.Y.). The instrument is a modified and miniaturized classical Van Slyke manometric method of gas analysis. Gases are released from a liquid sample, and by selective absorption of specific gases and changes in total volume, at a standard pressure, the amount of a specific gas can be determined.

Blood samples were taken by cardiac puncture. A 0.5 ml syringe that had been rinsed with heparin was used. All animals were lightly anesthetized with Metofane (Pitman-Moore) and given 0.4 ml of sterile physiological

saline I.P. immediately before blood sampling. The blood sample was maintained in an ice bath prior to tonometric equilibrium with known concentration gases.

The tonometric vessels were 3 ml conical bottom plastic beakers (Auto Analyzer, Technicon Instrument Corp.) fitted with rubber dropper bulbs so that gases could be injected via hypodermic needles. The elongate squeeze portion of the bulb was filled with fine glass wool-saturated with water so that incoming dry gases would be humidified. The excurrent port needle penetrated the dropper near the beaker lip and extended approximately one-half way into the beaker to assure good mixing of the residual and incurrent gases. All beakers were lined with a coating of silicon grease to present a hydrophobic surface on which to place the blood sample. Although 0.03 ml of blood was required by the analyzer, 0.05 ml of blood was placed in each tonometric vessel. Known concentration oxygen mixtures, varying from 10-90 mm partial pressure oxygen with 41 mm carbon dioxide and makeup nitrogen (obtained from and analyzed by Matheson Corp.) were passed through the tonometers in two 200 ml flushes separated by a 10-minute equilibrium period. Total equilibrium time was 20 minutes in a 37.0°C constant temperature incubator. During equilibration, the tonometers were rotated in a Bryan-Garrey blood pipette rotor. Before placing the blood samples in the microgasometer, they were covered with a 3 mm layer of de-aerated caprylic alcohol injected into the sealed tonometer.

All time schedules were rigidly observed to preserve a common temporal baseline. All microgasometric analyses were preceded by a daily reagent and instrument check via a determination of oxygen in air, and all blood sample determinations included a complete procedural check with a nitrogen-carbon dioxide blood equilibration.

Experimental values for oxygen content expressed a percent of maximum capacity of the blood, at the various concentrations of oxygen, were plotted on linear graph paper. A curve was fitted to the mean values (N=5, except P. californicus N=3), and the point at which half of the hemoglobin was saturated ($T_{\frac{1}{2}}^1$ sat) was determined.

It should be emphasized that all determinations were made on whole, undiluted and unbuffered blood at 41 mm CO₂ pressure. Thus the blood was kept as close to conditions existing in the living animal as practicable under experimental conditions, except for the inevitable aging of the sample during the several hours that elapsed during the determination of the dissociation curve.

RESULTS AND DISCUSSION

I. Hypoxic Hypothermia

Table I is a summary of the response of P. formosus to hypoxic induced hypothermia. Of the six animals run, one showed a spontaneous re-warming from a deep body temperature of 7.5°C. However, in four of the six animals, re-warming did not take place when the deep body temperature was between 10-11°C. One animal successfully re-warmed from 12.5°C several days later. This animal had a deep body temperature near 7°C for 14 hours, yet maintained its capacity to be re-warmed and live. Three of the four animals that showed no re-warming died before 15 hours from the time of restoration of normal atmosphere. This low temperature viability is apparently not directly associated with an immediate successful low temperature recovery as compared in F-597 and F-B, Table 1.

P. fallax response to hypoxia induced hypothermia is summarized in Table II. One animal was unable to re-warm from 16.6°C and was dead before 15 hours at 7°C. Four other animals could not re-warm between temperatures of 11.9-12.7°C. Two of these animals had previously re-warmed from 14.8°C to 14.3°C, respectively. After a series of progressively deeper hypothermic experiences, a P. fallax re-warmed from a temperature of 9.2°C (F-824, Table II), and there is strong evidence that this same animal would have been able to arouse from a temperature near 7°C, as noted in the remarks (Table II). This animal underwent an arousal from 9.2°C to normal temperature and, after a period, entered a spontaneous torpor. A spontaneous re-warming from this latter torpor only reached 17.8°C when the animal re-entered torpor and died before 12 hours. The point is that the rewarming processes were successful in raising the body temperature

Table I. HYPOXIC HYPOTHERMIA IN *P. FORMOSUS*

Animal	Date of Hypoxic Exposure	Method: * 1. Step 2. Single 3. Rebreath	Final O ₂ Conc %	T _B at Return to Normal O ₂ °C	Lowest T _B ** Attained °C	Time Lag to Rewarm	Max. Warming Rate °C/min	Total Time to Normal T _B min	Remarks
F-C female	13 May	1.	9.8	19.0	19.0	0	0.270	94	1. 300 mins required to warm to T _B 13°C. 2. T _B stabilized for 30 mins at 25°C, forming plateau in rewarming curve.
	31 May	1.	8.0	17.0	17.0	0	0.150	265	
	6 June	1.	5.0	9.0	7.5	20	0.103	515	
F-597 male	7 Aug	1.	5.6	12.5	12.5	0	0.139	270	1. T _B essentially stabilized at 10.8°C for 140 mins when air restored. T _B decreased to near T _A for 14 hrs. NO visible respiration, removed to room temp., rewarmed to normal body temp.
	9 Aug	1.	5.0	10.9	T _A = ~7°C	No Arousal	-	-	
F-595 male	3 Aug	1.	7.0	11.2	T _A = ~7°C	No Arousal	-	-	1. Cooling rate unaffected by restoration of air, dead before 15 hrs.
F-1060 female	8 Aug	1.	7.8	10.0	T _A = ~7°C	No Arousal	-	-	1. Dead before 15 hrs.
F-B female	5 June	1.	6.5	14.0	14.0	0.208	0.208	225	1. Cooling rate unaffected by restoration of air, dead before 15 hrs.
	7 June	1.	5.0	10.0	T _A = ~7°C	No Arousal	-	-	
F-748 female	1 June	1.	5.8	15.0	15.0	0	0.116	230	

* Step, single & rebreath refer to method of reduction of oxygen content in chamber; step = sequential reductions in increments; single = direct change to reduced level; rebreath = reduction by animal in a sealed chamber.

** T_B deep body temperature.

Table II. HYPOTHERMIA IN *P. fallax*

Animal	Date of Hypoxic Exposure	Method:*	Final O ₂ Conc %	T _B at Return Normal O ₂ °C	Lowest T _B ** Attained °C	Time Lag to Rewarm min	Max Warming Rate °C/min	Total Time to Normal T _B min	Remarks
F-824 female	24 Aug	1.	8.0	25.7	25.2	5	0.172	70	1. Total time not available, as terminated before T _B stabilized.
	29 Aug	1.	7.5	23.7	23.7	0	0.399	41	
	31 Aug	1.	5.0	18.6	18.6	0	0.433	-	
	1 Sept	3.		13.8	13.8	0	not deter.	not deter.	
	12 Sept	2.	5.5	13.8	13.8	0	0.273	160	
	14 Sept	2.	5.0	11.9	10.5	30	0.356	235	
F-617 male	18 Sept	2.	5.0	9.2	9.2	0	0.386	190	1. After 12 hrs of normal body temp., animal entered a spontaneous torpor for 9 hrs. (T _B = ~7°C). A spontaneous arousal occurred but animal only warmed to 17.8°C & again cooled to ambient. It died before 12 hrs. in this torpor.
	6 Sept	3.		12.5	T _A ~7°C	no arousal	-	-	
F-869 female	25 Aug	1.	8.5	12.7	T _A ~7°C	no arousal	-	-	1. Rate of cooling after air restored less than in hypoxic mixture. 2. Dead before 3 hrs after low T _B
	14 Aug	3.		15.8	14.8	17	0.400	not deter.	
F-4 male	15 Aug	1.	7.5	11.9	T _A ~7°C	no arousal	-	-	1. Cooling rate retarded after air restored. 2. Dead before 15 hrs. after low T _B

Table II (Continued)

Animal	Date of Hypoxic Exposure	Method*	Final O ₂ Conc %	T _B at Return Normal O ₂ °C	Lowest T _B ** Attained °C	Time Lag to Rewarm min	Max Warming Rate °C/min	Total Time to Normal T _B min	Remarks
F-867 male	21 Aug	3.		26.0	26.0	0	not deter.	-	1. Rewarm record erratic, data not good.
	23 Aug	1.	6.8	16.6	T _A = ~7°C	no arousal	-	-	1. Cooling rate retarded after air restored. 2. Dead before 15 hrs after low T _B
F-829	5 Sept	1.	6.5	16.8	16.8	0	0.222	135	1. After 3 hrs at T _B 9°C, resp. rate 12/min. After 18 hrs, no visible resp., warmed to 15°C, respiration erratic. EKG = 40. 2. Retained at room temp., dead before 15 hrs.
	8 Sept	2.	5	14.7	14.3	9	0.300	180	
	11 Sept	2.	4.8	12.1	T _A 7°C	no arousal	-	-	

* Step, single & rebreath refer to method of reduction of oxygen content in chamber; step = sequential reductions in increments; single = direct change to reduced level; rebreath = reduction by animal in a sealed chamber.

** T_B deep body temperatures

up out of the critical range. The decline and eventual death of the individual were probably associated with the depletion of energy stores during the immediately preceding thermal stresses.

It is difficult not to speculate on the effect of the previous hypothermic experiences of the animal that aroused from 9.2°C (and from 7°C, also). Would this animal have aroused from these low temperatures if it had been exposed to them without "benefit" of the previous arousal? There is evidence that the animal probably had gained some advantage through these trial exposures, an "adaptation to hypothermia." It has been demonstrated that white rats previously experiencing hypothermia have an improved operant behaviour with regard to the acquisition of external heat. These experienced rats acquired an instrumental technique of artificially warming faster and from a lower body temperature (25°C vs 29°C) than the hypothermic novice (4).

This "adaptation to hypothermia" may be the laboratory equivalent of the naturally occurring "test drops" observed in animals preparing for hibernation. A related phenomenon has been observed in P. longimembris, in that periods of torpor increase in length to the maximum value of 72 hours (5). There is a gradual increase in the length of a naturally occurring torpor, and appears to be a "temporal" adaptation to the extended hypothermic state.

The significance of the conditioning to hypothermic states may be important to the definition of the zone of response available to a particular species, but it seems to be unimportant in bridging the responses between potential hibernators and obligate homeotherms. It is highly improbable to think that a highly experienced white rat could assume the responses of even a novice facultative homeotherm in a hypothermic situation.

The data thus far acquired on members of the genus Perognathus indicate that the basic response of the ability to spontaneously re-warm from artificially induced low body temperatures is present in P. longimembris, P. formosus, and P. fallax. The lowest temperature at which successful re-warming has been observed is: 9.1°C, 7.5°C and 7.5°C, respectively, at

an ambient temperature of 7°C. This level of response places them well into the range occupied by classical hibernators, i.e., ground squirrel arousal at 11°C vs rat at 23°C (6).

Specific experimentation with regard to extended stay at hypothermic temperatures compatible with survival was not undertaken, but several informative observations were made. One P. formosus was noted to survive re-warming after 14 hours at 7.5°C. In contrast, P. fallax appears to have a shorter hypothermic existence. Although one was noted to be alive after 18 hours at 7.5°C, and remained alive in the re-warming process to 15°C, it died within 15 hours. Another one died within 3 hours after low body temperature was reached.

Earlier work had indicated that P. longimembris probably had a maximum induced hypothermic limit compatible with survival of near 36 hours (3). However, since that time, successful restoration of normal body temperature has been made after 86 hours at a body temperature near 7.5° C. Three healthy survivors of 70, 72 and 86 hours have been maintained in the laboratory for a number of months. This upper value well surpasses the maximum duration of a natural hypothermic state so far observed in the laboratory under optimum conditions as to time of year, isolation and environmental temperature (5).

II. Hemoglobin - Oxygen Affinity

The binding of oxygen to the hemoglobin molecule has certain characteristics that are described by the so-called oxygen dissociation curve of blood. The sigmoid shape of a typical dissociation curve is associated with the interaction of the iron contained in the 4 heme portions of the hemoglobin molecule. The most important single characteristic that is used to compare the dissociation curves of various animal species is the point (in concentration of oxygen expressed in mm Hg) at which half of the hemoglobin is saturated with oxygen ($Hb = HbO_2$). This value of T_{50} or $T_{\frac{1}{2}}^{sat}$ is a measure of the relative degree of the tendency of the hemoglobin to join with oxygen in a loose association.

The affinity of the hemoglobin for oxygen is significant in two processes (a) the uptake of oxygen in the lungs and (b) the unloading of oxygen in the tissues. The examples of the shifting of the dissociation curves to the left (more affinity) of certain mammals at high altitude permit the hemoglobin to become fully saturated with oxygen at a partial pressure at which the blood of other mammals is only partially saturated (7). Another example of the left displacement is the fetal blood as compared to that of the maternal organism (8). Similar relationships have been described for the developing chick and for the larva of the bullfrog as compared to the adult (9,10).

All of these animals have in common an environment of relative oxygen scarcity, which is being met by a blood adapted to take on a full load of oxygen at a lower oxygen pressure.

However, it has been documented that there is a characteristic shift of the curve to the right with the diminishing size (weight) of the animal which seems to be an adaptation to the metabolic need for oxygen and, therefore, directly related to the unloading of oxygen in the tissues (11). It has been calculated that oxygen must be supplied to the tissues of a mouse 15 times faster than to a horse. This increased need for high oxygen is in part met in the mouse by decreasing the diffusion distance from capillary to cell (increased capillaries per square millimeter cross section) and maintaining a higher difference in oxygen tension at the capillary and the cell (an increased capillary level).

Recent work on various species of rodents, as to position of dissociation curve and species ability to extract oxygen from hypoxic atmosphere tends to confirm the hypothesis of size-oxygen affinity relationship (12, 13). Several notable exceptions existed and were explained on the basis of certain ecological factors in these species.

The effect of pH changes and the shifting of the dissociation curve indicates that the smaller the animal the greater the sensitivity to pH shift (14). It has also been observed that a decreasing oxygen affinity is accompanied by an increasing Bohr effect (15).

It has been hypothesized that mammals that hibernate have hemoglobin which bind oxygen at low pressures. This suggestion is complicated by the fact that temperature exerts a strong influence on the position of the dissociation curve. Human hemoglobin binds oxygen so tightly at low temperatures that it becomes almost useless as a carrier at 15°C (shifted to the left).

It was the aim of this phase of the present study to determine the dissociation curves for a number of species of pocket mice (Perognathus) and evaluate the results in the light of present knowledge concerning response to low body temperatures, natural torpor and response to ionizing radiation.

Dissociation curves for 8 species of pocket mice are presented in Figure 1. All dissociation curves exhibit the characteristic sigmoid shape to varying degrees. Table III is a tabulation of the $T_{1/2}$ sat values for the species under consideration.

Table III Hemoglobin half saturation values for 8 species of pocket mice (Perognathus)

<u>Species</u>	<u>No.</u>	<u>Weight (grams)</u>	<u>$T_{1/2}$ sat (mm Hg)</u>
<u>P. longimembris</u>	5	10.5 <u>+0.9</u>	47
<u>P. amplus</u>	5	16.9 <u>+3.4</u>	35
<u>P. formosus</u>	5	20.7 <u>+1.5</u>	56
<u>P. parvus</u>	5	22.7 <u>+6.7</u>	35
<u>P. pennicillatus</u>	5	25.8 <u>+1.9</u>	41
<u>P. fallax</u>	5	26.2 <u>+3.4</u>	50
<u>P. californicus</u>	3	30.4 <u>+2.4</u>	44
<u>P. baileyi</u>	5	41.9 <u>+9.8</u>	44

VERTICAL LINES INDICATE RANGE
 HORIZONTAL LINE INDICATES MEAN
 RECTANGLES ENCLOSE INTERVAL $\bar{X} \pm 1\sigma$

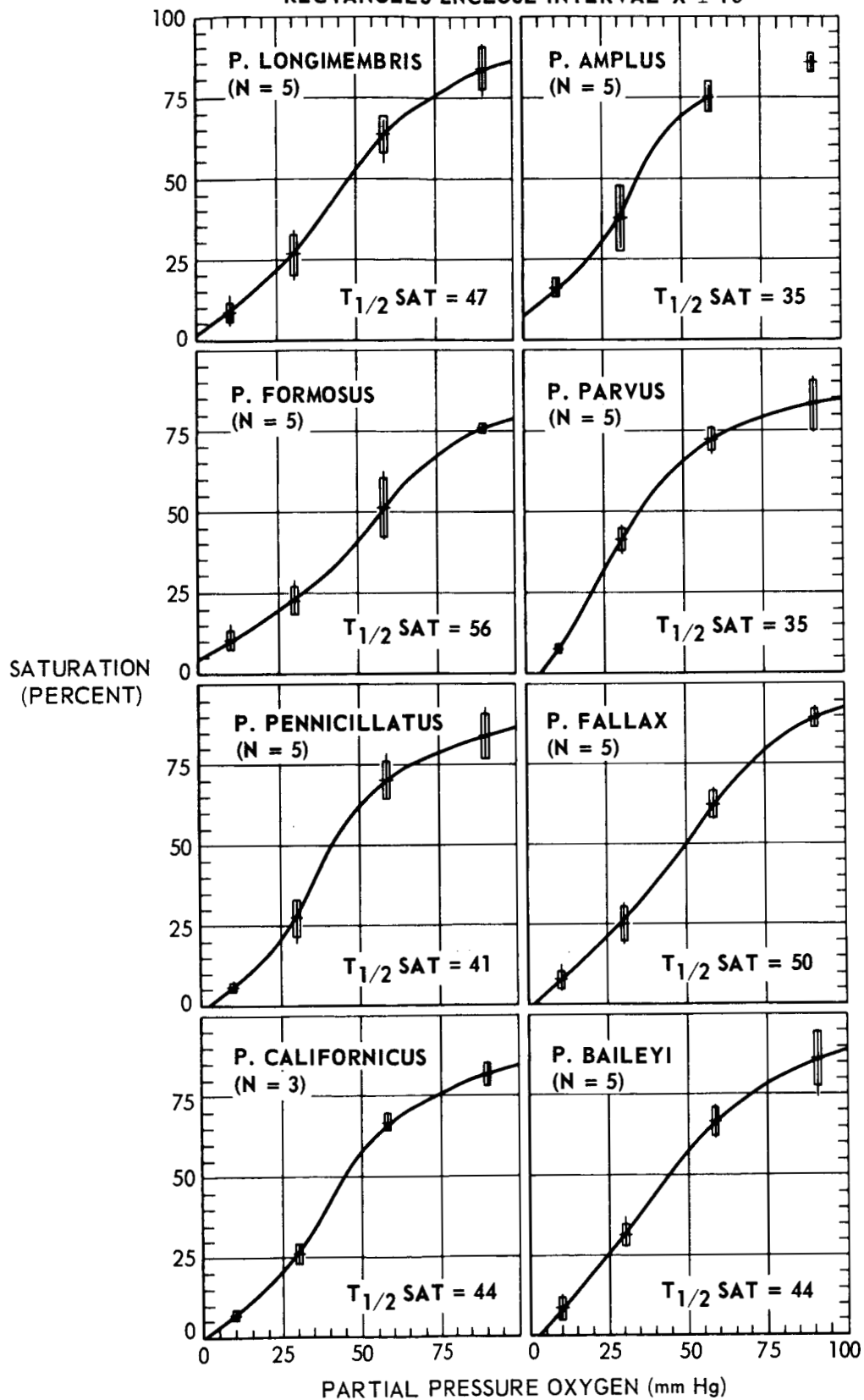


Figure 1. Oxygen dissociation curves.

It will be noted from Table III that there was only a 4 fold change in body weight from the lightest to the heaviest species; yet there was a 60% change in the $T_{\frac{1}{2}}$ sat values (35-56). It would seem that this group of animals generally does fall within the expected range of $T_{\frac{1}{2}}$ sat values, from 10-40 gm should be 41 to 46 mm Hg (11). But several values fall outside the range with a $T_{\frac{1}{2}}$ of 35 mm equal to about 600 gm. The higher values of 47, 50, 56 agree fairly well with experimental values of white mouse, deer mouse, house mouse, kangaroo rat (49, 50, 52, 53, respectively, Table IV)(11, 13).

The prairie dog has blood that has a great affinity for oxygen ($T_{\frac{1}{2}}$ sat = 22 mm Hg) and it has been hypothesized to be associated with its fossorial nature which may present hypoxic situations. However, the same paper ignores the kangaroo rat which is equally fossorial with a $T_{\frac{1}{2}}$ sat = 53 mm Hg. All of the pocket mice in the present study are fossorial and some spend considerable periods of time underground. The interpretation of the fossorial nature and high blood affinity for oxygen would be more complete if it were noted that prairie dogs are gregarious and live in colonies, as opposed to the other species which are solitary. It would seem that a social underground existence could result in hypoxic conditions.

As of this date, only three species have been adequately tested as to response to hypoxia and cold, with emphasis on re-warming from hypothermia. P. longimembris, P. fallax and P. formosus (see first section for details) all have relatively high $T_{\frac{1}{2}}$ sat values, 47, 50, and 56, respectively. All three species are able to re-warm from deep body temperatures below 10° C. It has been stated that P. californicus is unable to re-warm from spontaneous torpor below 15° C (2). This species has been determined to have a blood which has a greater affinity for oxygen than those that can re-warm from a low body temperature.

It may be significant to note that the relatively high $T_{\frac{1}{2}}$ sat values of those animals that can re-warm from low temperatures may be an advantage at low temperatures. If the dissociation curve is shifted to the left with low temperatures, those animals that require a relatively high partial pressure of oxygen (high $T_{\frac{1}{2}}$ sat) may have a dissociation curve that is still in the usable range at low temperature. This is opposed to those that have a high binding capacity (low $T_{\frac{1}{2}}$ sat) at normal body temperatures but are unable to utilize the transported oxygen at low temperature because it is bound tightly to the hemoglobin.

Table IV

A COMPARISON OF $T_{1/2}$ SAT VALUES OF VARIOUS RODENTS AND LAGOMORPHS

	Weight gm	$T_{1/2}$ SAT	
		Schmidt- Nielsen and Larimer 1958	Hall 1966
1. white rat (<u>Rattus norvegicus</u>)	245	33	38
2. hamster (<u>Mesorcricetus auratus</u>)	88	29	29
3. cotton rat (<u>Sigmodon hispidus</u>)	162	40	39
4. guinea pig (<u>Cavia porcellus</u>)	375	34	30
5. white mouse (<u>Mus musculus</u>)	30	49	-
6. house mouse (<u>Mus musculus</u>)	18	-	52
7. deer mouse (<u>Peromyscus sp.</u>)	50	50	-
8. kangaroo rat (<u>Dipodomys merriami</u>)	47	-	53
9. jack rabbit (<u>Lepus californicus</u>)	2,045	-	23
10. cottontail rabbit (<u>Sylvilagus floridanus</u>)	2,242	-	31
11. prairie dog (<u>Cynomys ludovicianus</u>)	1,200	-	22

The resistance to ionizing radiation exhibited by several members of this genus, P. longimembris, P. formosus and P. parvus, apparently cannot be correlated directly with the oxygen transport system as delineated by the dissociation curve of whole blood. These resistant species of pocket mice fall into the extremes of the $T_{\frac{1}{2}}$ sat values 35 and 56, with the most resistant species, P. longimembris, in between.

SUMMARY

1. The pocket mice, P. longimembris, P. formosus and P. fallax are able to re-warm from artificially induced hypothermia at a level well into the range occupied by a classical hibernator, i.e., ground squirrel.
2. The $T_{\frac{1}{2}}$ sat values of the oxygen dissociation curve of whole blood determined on eight species of pocket mice generally fall within the expected range based on size. The blood of P. parvus and P. amplus have a greater affinity, and P. formosus and P. fallax have a lesser affinity for oxygen than predicted.
3. Variation of hemoglobin-oxygen affinity at the species level is as great as the variation in intraspecific comparisons.
4. At this time, there appears to be no direct correlation between high blood-oxygen affinity and ability to re-warm from induced deep hypothermia in pocket mice.

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