PRODUCTION AND LIFE SPAN OF ERYTHROCYTES
DURING HIBERNATION IN THE GOLDEN HAMSTER

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ABSTRACT

One day following radioiron injection, the concentration of the isotope in erythrocytes of control hamsters was 14.1 times the concentration in hibernators' erythrocytes during the second day of hibernation. During this same period, hibernators' plasma contained 4.62 times the control concentration of radioiron. At definite times after tagging, the activity of chromium^{51}-labeled erythrocytes was measured in warm- and cold-exposed animals and in hamsters after measured periods of hibernation. In the active animals, the potential erythrocyte half-life was approximately 40 days; the effective half-life was about 15 days. Retardation of senescence and the virtual absence of random destruction in erythrocytes of hibernating hamsters increased the potential erythrocyte life to approximately 160 days. Both the minimal metabolic activity of the animal and the retardation of intrinsic senescent processes in erythrocytes were considered to be responsible for the diminished erythropoietic stimulus in hibernating hamsters. Radioiron transport across the cell membrane, its incorporation into hemoglobin, and mitotic activity of narrow erythroid elements were component processes of erythropoiesis which were probably retarded.

PRODUCTION AND LIFE SPAN OF ERYTHYROCYTES DURING HIBERNATION IN THE GOLDEN HAMSTER[*]

There has been much research and speculation concerning the environmental and intrinsic factors which alter the aging of both the individual organism and single cells (Cowdry, 1952). Although the aging of mammalian erythrocytes has been examined in some detail, there has been little work on the effect of cold on the life span of red blood cells in the intact animal. Hibernating mammals offer an opportunity for such a study. Observations that no apparent anemia occurred during deep hibernation of the golden hamster (Mesocricetus auratus), although cell division was depressed, suggested a mechanism involving altered cellular longevity. The general depression of most physiological processes at body temperatures close to 0° C and Brace's (1953) observations on one hibernating marmot also implied that the erythrocyte life was extended.

In this study, observations were made using radioisotopes to describe the life span of erythrocytes in control golden hamsters, in hamsters in deep hibernation, and in hamsters awake in the cold before and after hibernation. By measuring the quantity of injected radioiron which appeared in erythrocytes in 1 day, the rate of erythropoiesis was estimated, while the disappearance of radioactive chromium from tagged erythrocyte populations was used to indicate their life spans.

MATERIALS AND METHODS

Golden hamsters, Mesocricetus auratus, employed as controls were kept in rooms at $22^{\circ} \pm 2^{\circ}$ C, while experimental animals were maintained in cold rooms at $8^{\circ} \pm 2^{\circ}$ C. All animals, housed singly in cages, were given Purina laboratory chow and water. Excess food and wood shavings for nests were supplied to hamsters in the cold room to induce hibernation (Lyman, 1954). Small wood chips were placed on the backs of hibernating animals, and the method of Lyman, et al. (1957) was used to determine the length of hibernation. The body temperature of hibernating hamsters has been shown to be less than 0.5° C above the environmental temperature (Lyman and Chalfield, 1955).

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[*]Submitted for publication 1 June 1960

Radioiron, $Fe^{55-59}Cl_3$, was obtained from Union Carbide Nuclear Co. with specific activities in 4 shipments of 28.23, 19.41, 4.34 and 2.48 millicuries per milligram (mc/mg) iron. Ferric ammonium citrate solutions were prepared (Peacock, et al., 1946), and 0.684, 1.368 or 2.736 μg iron in 0.2 ml were injected into each animal.

Sodium radiochromate (Rachromate), $Na_2Cr^{51}O_4$, was obtained from Abbott Radio Pharmaceutical Laboratories with specific activities ranging from 1.752 to 58.0 mc/mg chromium. Injections of 0.2 ml containing 20 μc were made into each animal.

Radioiron was administered by cardiac puncture to all hamsters with the exception of hibernators. The intubation technique of Still and Whitcomb (1956) was modified so that hibernators could be injected without arousal through a polyethylene tube leading into the aorta. Hibernating hamsters were aroused and anesthetized with an intraperitoneal injection of pentobarbital sodium (9 mg in 1 ml). The polyethylene tube (PE 10 or smaller) was inserted into the aorta slightly posterior to the renal arteries, sutured in place, and led to the dorsal surface of the hamster where it projected from the skin between the scapulae. To block blood flow and to prevent blood clots, heparin-saline solution (66.5 USP units/ml) was introduced into the tube which was then closed distally with a knot. Forty-thousand units procaine penicillin were injected intramuscularly, and, following recovery from anesthesia, the hamsters were returned to the cold room where some of them eventually hibernated. After 24 hours of hibernation, 0.2 ml of the radioiron solution was injected through the polyethylene tubing. An insignificant amount remained in the tube.

Injections of Rachromate were all made by cardiac puncture. Torpid hamsters injected with Cr^{51} either were allowed to return to hibernation or were moved to a warm room.

With the exception of hibernating animals, ether anesthesia was employed during injections and sample removals. Number 26 needles were used, and approximately 25 units of heparin served as anticoagulant in obtaining 0.2 to 0.4 ml samples of whole blood.

Both hematocrit determinations and radioactivity counting of cells were done in 105 mm Wintrobe Hematocrit tubes after the blood had been centrifuged for 45 minutes at 1500 rpm in an International Clinical Centrifuge. Plasma was pipetted from the Wintrobe tubes and counted separately.

The gamma radiations were detected in a well-type NaI scintillation counter manufactured by Baird-Atomic, Inc. (Cambridge, Mass.) which

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was similar to No. 810. Auxiliary equipment (Baird-Atomic, Inc.) included a nonoverloading amplifier (No. 215), single channel differential pulse height analyzer (No. 510), and a scaler and power supply (No. 1032). The counting efficiency for this system with a base line setting which included most of the pulses generated by Cr^{51} was 26%.

Both Cr^{51} and Fe^{59} standards were prepared at the beginning of each experiment and counted with each blood sample to correct for radioactive decay of the isotope and for slight variations in counting efficiency over a period of time. The background was measured for 50 minutes. The total counts recorded in the samples were reproducible with a standard deviation of ± 5% or less, except in the total Fe^{59} counts in hibernators' samples where the standard deviation was from 10 to 20%.

Twenty-four hours after the injection of Fe^{59}, blood was removed from hibernating animals, and two methods were used to correct for the high radioactivity in the trapped plasma. In the first, a measured amount of radioiron was incubated with hamster whole blood for 1 day at 5° C. No radioiron could be detected in the hemoglobin of the red cells. After three washings of the erythrocytes with acid citrate dextrose (ACD), National Institutes of Health Formula A, the radioactivity was determined and represented that of the trapped plasma. This percentage was subtracted from the radioactivity of the hibernators' washed erythrocytes. The second method involved washing the red cells with excess ACD containing 0.01% nonradioiron as carrier until the supernatant counts did not exceed the background. It was assumed that no radioactivity remained then in the trapped plasma, and the red cells could be counted with no correction for trapped plasma.

In determining radioiron uptake, the corrected sample counts were divided by the observed erythrocyte volume and corrected for body weight to produce counts per minute (cpm)/ml red blood cells.

The percentage of the total injected iron activity which appeared in circulating erythrocytes in control hamsters was calculated, utilizing the blood volume data of Lyman, et al. (1957), and inserting it into the equation: Max. % uptake = animal wt. x $\overline{0.077}$ x ((Max. cpm/ml)/total cpm injected) x 100. It should be emphasized that these were average values and were considered approximations which could be used in determining the optimum radioiron injection dosage.

The corrected Rachromate sample counts were divided by the observed erythrocyte volume and multiplied by a factor to produce 9000 cpm/ml on

the third day following Cr^{51} injection. Blood samples were taken initially 3 days after Cr^{51} injection, and at approximately 2-week intervals thereafter. To determine the peak of Cr^{51} uptake in later experiments, samples were procured 6 hours and 1, 2, and 3 days following the injection.

The Rachromate activity of erythrocytes from hibernating hamsters was graphed in the following manner. The cpm/ml red cells of each hibernator's sample was plotted on the graph established for the warm-room controls. The vertical distance from this plot to the control curve was measured. This same distance was measured above the control curve at the point which corresponded to the total number of days which the animal had hibernated, and a point was placed at this position.

All results are expressed as the "apparent" erythrocyte life span since no correction was made for elution, the random loss of Cr^{51} from the red cells.

RESULTS

Radioiron

Preliminary experiments indicated that injections of either 1.368 or 2.736 μg iron resulted in easily detectable amounts of radioactivity appearing in hamster erythrocytes in 1 day (see Table I). The radioiron uptake curves in most cases rose rapidly during the first 6 days, reached a maximum between 6 and 12 days, and thereafter, slowly declined over 60 to 65 days, which was as long as samples were taken. No attempt was made to utilize Fe^{59} to elucidate the erythrocyte life span.

Although intraperitoneal injections would have been less difficult, experiments showed that both the maximum uptake and the rate of uptake of radioiron by erythrocytes was less ($P < 0.01$) than with cardiac injections (see Table I). Therefore, radioiron was introduced directly into the blood stream.

The concentration of Fe^{59} appearing in erythrocytes of control hamsters 1 day following injection was 14.1 times that found after the second day of hibernation. On the other hand, 4.62 times the control value of radioiron was retained in the hibernators' plasma during this same time (see Table I).

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TABLE I

RADIOIRON VALUES IN CIRCULATING ERYTHROCYTES AND PLASMA 24 HOURS FOLLOWING ISOTOPE INJECTION

	Warm-room animals. Radioiron injected via cardiac puncture.		Warm-room animals. Radioiron injected intraperitoneally.		Hibernators. Radioiron injected via polyethylene tube.	
	cpm/cc RBC	cpm/cc Plasma	cpm/cc RBC	cpm/cc Plasma	cpm/cc RBC	cpm/cc Plasma
	871	720	675	237	14	2380
	366	498	540	212	166	2020
	454	810	318	214	104	1140
	1480	108	380	175	57	805
	1265	55			17	910
	740	304			57	1280
	1440	158				
	1300	109				
	547	137				
	1270	180				
Mean	973	308	478	210	69	1423
S. E. ±	134	87	81	13	24	258

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Both differences were highly significant (P > 0.01).

Rachromate Uptake

With injections of 20 μc Cr^{51}, the metallic chromium concentration administered to each animal ranged between 0.34 and 11.4 μg. Assuming an average red blood cell volume of 4.5 ml, this represented 0.075 to 2.5 μg chromium/ml erythrocytes which was well below 23.5 μg chromium/ml red cells which was the maximum concentration tested without affecting the half-time of red cell survival in the rabbit (Donohue, et al., 1955).

The average Cr^{51} uptake by the erythrocytes was 16.3% of the injection dose with a range of 4.62 to 28.0%. Data from 37 hamsters indicated that there was apparently no relationship between the amount of radioactivity taken up and the length of time required to reach the maximum. No direct or inverse relationship of the rate of Rachromate uptake to the rate of decline was found. In addition, the maximum Rachromate uptake was not dependent upon the total hemoglobin or hematocrit values determined in 15 hamsters.

Interpretations of Erythrocyte Life Span Data in Control Hamsters

Utilizing the analyses developed by Eadie and Brown, (1953) and Sheets, et al. (1951), the survival curve obtained was evaluated as one depicting slow random destruction at a constant rate plus normal loss of senescent erythrocytes.

The disappearance of Rachromate from circulating erythrocytes may be described by the equation

(1) $N = N_0(1-t/T)e^{-.693t/\gamma}$

where N = radioactivity count at time t after injection,

N_0 = radioactivity count at t = 0,

T = potential erythrocyte life span, and

γ = half-life of random destruction.

By trial and error, it was found that a mean life value, T, of 78.5 days and a half-life of random destruction, γ, of 22.5 days substituted

into equation (1) produced a theoretical curve which closely coincided with the experimental values (see Fig. 1).

The two constants may be evaluated:

$a = 1/T = 0.0128$ or 1.28% of the original number of tagged red blood cells destroyed per day due to senescence.

$b = 0.693T = 0.0308$ or 3.08% of the tagged red blood cells present destroyed per day as a result of random loss.

The random loss includes both physiological random erythrocyte destruction and elution of Cr^{51} from the cells. The mechanism of the loss through elution is unknown, but it has been discussed by Necheles, et al. (1953) and Ebaugh, et al. (1953) among others, and calculated to be approximately 1% of the remaining radioactivity per day.

A second analysis described by Eadie and Brown (1953) was applied to the data. Equation (1) may be rewritten

(2) $\ln N - \ln N_0(1-t/T) = -bt$

and various values for T may be assumed. Using these, plus the numerical equivalents of N and N_0 from the experimental data, -bt may be calculated. If the assumed value of T is correct, a straight line will be produced when -bt is plotted against t. The slope of the line is equal to b. The values of T, a, and b calculated previously were reconfirmed.

The mean potential half-life of the erythrocyte population was about 39 days, while the mean half-survival time or effective half-life was 15 days due to random loss.

Interpretations of Erythrocyte Life Span Data in Experimental Animals

The radioactivity of blood samples from 28 active, cold-exposed animals fitted the theoretical curve calculated for control animals. It was apparent that neither the rate of senescence nor that of random loss was altered.

In Figure 2, the replotted values of erythrocyte radioactivity during hibernation are shown. The line representing the mean red blood cell life span during hibernation was drawn after determining the points where the sum of the squares of the positive deviations equalled the sum of the squares of the negative deviations.
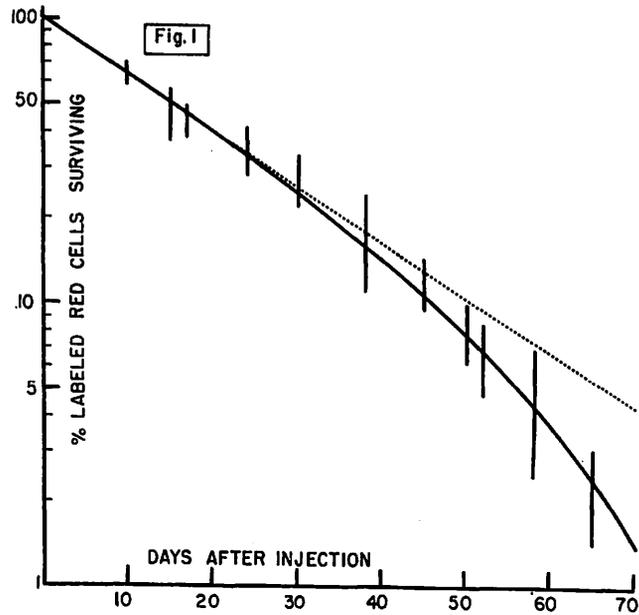
Figure 1. "Apparent" erythrocyte life span calculated from the radioactivity of over 125 blood samples taken from 43 control hamsters in the warm room. The dotted line represents a continuation of the initial straight solid line which is primarily a logarithmic decline in radio-activity. The ranges of 2/3 of the data representing the probable deviations of the means are superimposed on the solid line.
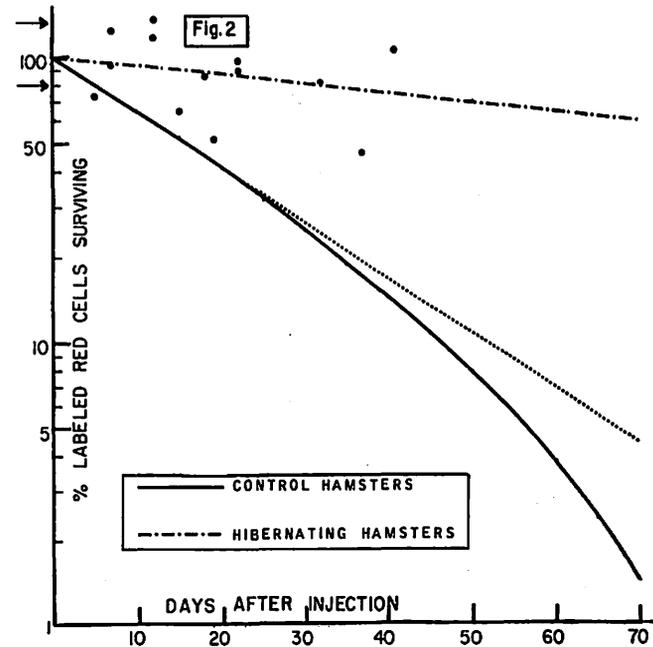
Figure 2. "Apparent" red blood cell life span of 12 hibernating hamsters. The solid line and dotted line are as in Fig. 1. The solid dots represent the replotted values of each hibernator's blood sample. The range delimited by the two arrows is that of the maximum uptake of Cr^{51} radio-activity in warm room control animals.

In order to elucidate the degree of random destruction during hibernation, various values of T were substituted into equation (2). None of these produced a straight line when -bt was plotted against t. However, the lines progressively approached one which would be parallel to the abscissa, and with T = 150 days, only slight deviations from a parallel line were seen at 40 and 45 days. Since Eadie and Brown (1953) have stated that if there is no random destruction, the line will be parallel to the abscissa (b = 0), these data suggested that random destruction was either not occurring during hibernation, or was at a minimum.

The line describing the experimental values may be drawn on arithmetical paper, and its intercept with the abscissa will be a T value equal to the mean life span of the erythrocytes. This is approximately 160 days during hibernation compared with 78.5 days in the controls.

The two constants may be calculated:

a = 1/T = 0.00625 or 0.625% of the original number of tagged red blood cells destroyed per day due to senescence.

Values for b were determined by substituting experimental values for N and N_0 at 10-day intervals from day 10 to day 50 and the calculated equivalent of a, 0.00625, into the equation

(3) $N = N_0(1-at)e^{-bt}$

which is given by Sheets, et al. (1951) in their analysis. A range was found from 0.00332 to 0.00497 with a mean of 0.00425 or 0.425% of the tagged erythrocytes present destroyed per day. Due to the error inherent in this method, this number was probably not significant, and it can be assumed that physiological random destruction of erythrocytes and Cr^{51} elution were essentially absent during hibernation of the golden hamster.

A relation between temperature and Cr^{51} elution has been demonstrated previously, for no loss of Cr^{51} occurred when tagged red cells were stored at approximately 5° C in ACD (Ebough, et al., 1953). The similarity of these in vitro results to in vivo effects during hibernation is apparent.

No significant differences in the rate of erythrocyte destruction immediately following long periods of hibernation were seen when the experimental results from six animals were compared to the theoretical curve depicting "apparent" erythrocyte life span in control hamsters. These results further implied that senescent processes had not been occurring at the

control level during hibernation, for red blood cells which were present for as many as 45 hibernating days were not destroyed any more rapidly when the hamster awakened.

Plasma Rachromate Radioactivity

A very rapid decline in plasma radioactivity was observed during the first 5 days following Rachromate injection during which excess chromium which had not tagged erythrocytes was eliminated. Thereafter, the radioactivity level diminished slowly, and the chromium counted was presumably that which was freed after tagged red blood cells had been destroyed. Approximately 2.5% of the original plasma radioactivity persisted for as long as 60 days, after which measurements were not continued.
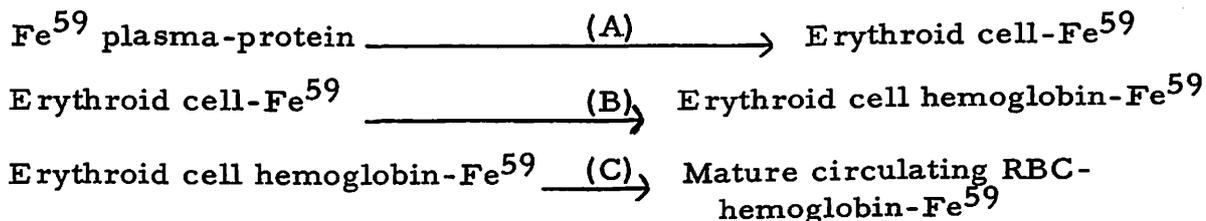
DISCUSSION

Erythropoiesis

The elucidation of the rate of erythrocyte production during hibernation has been approached indirectly. Raths (1953) described a retardation in both hemoglobin and erythrocyte regeneration following the bleeding of hibernating European hamsters. However, only two of the six hamsters studied returned to hibernation after bleeding, and they did so after 12 and 22 days had elapsed. Confirmation of this phenomenon was made by Lyman, et al. (1957). In data from 18 golden hamsters which hibernated at least 2 of 4 days after bleeding, a decreased reticulocyte response and rate of hemoglobin synthesis was described, and this response was increasingly depressed as the percentage of hibernating time increased. Observations in both papers that some reticulocytes were present in hamster blood, regardless of duration of hibernating time, would substantiate the evidence that erythrocyte production does not cease entirely during hibernation, but is merely reduced.

Further evidence for depressed erythropoiesis is found in the histological treatments of Lyman, et al. (1957) and Brace (1952). The former authors observed a decrease in the number of myeloid cells in both spleen and bone marrow during hibernation of the golden hamster, while the latter saw no erythropoietic activity in the bone marrow of the marmot after 4 and 8 weeks of hibernation.

The converse of these experiments or the elevation of the temperature of fatty bone marrow was described by Huggins and Blocksom(1936). The distal tail bones of normal rats which contained fatty bone marrow showed erythropoietic activity when the temperature was elevated to that of the bones containing red bone marrow.

One may conclude that, providing there is a constant erythropoietic stimulus, red blood cell production is directly proportional to the temperature of the bone marrow.

It should be pointed out that the amount of radioiron which appeared in mature circulating erythrocytes in the hibernating hamster was not expected to be a direct measurement of, but should be proportional to, red blood cell production. The path of radioiron from injection to its appearance in the mature erythrocyte may be illustrated:

Fe^{59} plasma-protein $\xrightarrow{\text{(A)}}$ Erythroid cell-Fe^{59}

Erythroid cell-Fe^{59} $\xrightarrow{\text{(B)}}$ Erythroid cell hemoglobin-Fe^{59}

Erythroid cell hemoglobin-Fe^{59} $\xrightarrow{\text{(C)}}$ Mature circulating RBC-hemoglobin-Fe^{59}

(A) = transport across the cell membrane; (B) = incorporation of Fe^{59} into hemoglobin; and (C) = mitotic activity and maturation. It is evident that the movement of radioiron could be blocked at (A), (B), or (C) or at all three steps. Since 4.62 times the control quantity of Fe^{59} was retained in the hibernators' plasma during 1 day of hibernation, one might suspect that the block was at (A). A significant decrease in adenosine triphosphate (ATP) in hibernators' erythrocytes (Brock, in press) suggests that less energy is available to the cell. This may also be true of erythroid elements in the bone marrow. Since accumulation of iron by the erythroid cells is probably an active process, some source of energy such as ATP should be available for its continued function. The decline in ATP during hibernation may partially impede (A).

The possibility that blocks at (B) or (C) might be responsible for the retention of activity in the plasma is not eliminated. A decreased incorporation of Fe^{59} into hemoglobin or a reduced mitotic rate in the hematopoietic tissue as postulated by Lyman, et al. (1957) would serve to slow reactions at (A), (B), and (C). Although the concentration of radioiron in the erythrocytes of the controls was 14.1 times that found in the hibernators', the concentration of radioiron in the plasma was only 4.6 times higher in

the hibernating animals. Thus, a large amount of the injected iron is unaccounted for in the hibernating animal, and it is natural to suspect that it is trapped in the erythroid elements of the bone marrow. This would indicate a block at (B) or (C), or both.

A retardation in the rate of cellular growth and proliferation during hibernation has been demonstrated in the seminal vesicles and heterologous and homologous tumors of golden hamsters (Lyman and Dempsey, 1951; Patterson, et al., 1957). A similar depression has been described in torpid marmots, hedgehogs, and ground squirrels (Hansemann, 1898; Sarnat and Hook, 1942; Smith and Grenan, 1951; Brace, 1952). The extrapolation of these results to erythroid tissue is feasible, and it suggests that the maturation of erythrocytes including mitosis during their development into circulating red blood cells is retarded during hibernation of the golden hamster.

Erythrocyte Longevity

Alterations in the red blood cell life span in a hibernating animal were described by Brace (1953), who employed C^{14} to label hemoglobin. One control marmot exhibited a mean erythrocyte life span of 36 ± 2 days. Cold exposure appeared to decrease the rate of senescence which was further depressed during hibernation. In comparing data from two marmots, both maintained at 4.5° C for 82 days, but one hibernating for 24 days, the difference observed in erythrocyte mean life span was not significant (P > 0.05); the red blood cell life span of both animals, however, was significantly different from the warm-room control marmot. The statement made by Brace that "conditions which tend to induce hibernation...effect a prolongation of the life span of the red cells" does not describe the situation in golden hamsters where there was no discernible difference in rate of erythrocyte destruction of hamsters awake in the cold before a period of hibernation compared to those in the warm.

In addition to isotope studies, histological evidence also points to a depression in the rate of erythrocyte destruction during hibernation. Mann and Drips (1917) examined the spleens of active and hibernating 13-lined ground squirrels and observed what appeared to be a decrease during hibernation in the number of phagocytic endothelial cells which contained red blood cells or blood pigment.

Environmental temperature effects upon the mean life span of erythrocytes maintained in vitro also have been studied. When red cells are stored

at 4° C in nutrient solutions, a large percentage of them will survive after injection into a recipient for a duration of time equal to their normal life span. Thus, a prolongation of their life for a period of time corresponding to the length of storage has been effected (Gabri , et. al., 1956). More dramatic results were obtained with storage in glycerol at -79° C (Chaplin, et al., 1956). About 80% of the red cells remained viable after 21 months of storage, an extension of the erythrocyte life for a period 5 times the mean life span.

Brace and Altland (1955) used radioactive carbon to label hemoglobin of box-turtle erythrocytes, and the radioactivity did not diminish for 11 months while the animals were maintained at room temperature. Although the turtle erythrocytes are nucleated, this is in vivo substantiation of the results of red cell storage experiments.

In the erythrocytes of the golden hamster, intrinsic aging processes are retarded when the cell's environmental temperature is lowered. One might extrapolate to this situation the statement which Bourliere (1954) made in comparing aging processes in poikilothermic and homeothermic animals, "the difference would be more a difference of tempo than a difference of mode." Yet, one may question whether cellular metabolism passively decelerates or actively adapts to the new environment, with the coincident depression of senescent rate.

The Humoral Regulation of Erythropoiesis

In his discussion of the erythropoietic stimulating factor (ESF), Gordon (1959) discussed the contemporary hypothesis that it is the relation between the oxygen tension of the blood and the oxygen demand by the tissues which determines the rate of ESF production and, thus, erythropoiesis. Consideration of the ESF may explain some of the alterations in erythropoiesis and red blood cell counts observed during cold exposure and hibernation in golden hamsters.

Analyses of the erythrocyte picture in the golden hamster (Lyman, et al., 1957; Suomalaenen and Granström, 1955) showed that the red blood cell counts expressed as million/mm^3 were not significantly different in hamsters in the cold in comparison to those in the warm. The red blood cell count of hibernating hamsters, however, was significantly higher than that of warm-room animals. By recalculating the results of Lyman, et al. (1957) and expressing them as total red blood cell volume, the following picture was produced: the total red blood cell volume of warm-room hamsters was

4. 27 ml; an increase to 5. 32 ml was incurred in hamsters awake in the cold; the volume decreased to 4. 44 ml after about 30 days of hibernation.

The increased red cell volume of cold-acclimatized hamsters may be due to the stimulation of erythropoiesis, while the rate of erythrocyte destruction is not altered. The elevated oxygen consumption of these animals (Lyman, 1948), creating an oxygen demand, stimulates ESF production. As the animal enters and remains in hibernation, the oxygen demand diminishes. This, plus the effect of cold, is reflected in a declining rate of erythropoiesis. Indirect evidence for a decrease in ESF is the observation of a depressed reticulocyte response following the bleeding of hibernating hamsters (Lyman, et al., 1957). The depressed rate of erythrocyte destruction in the hibernator probably exceeds the slow rate of erythropoiesis and accounts for the decrease in red cell volume.

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