UCRL-9862
Research and Development UC-48 Biology and Medicine
TID-4500 (16th Ed.)

UNIVERSITY OF CALIFORNIA
Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

RED CELL MASS AND LIFE SPAN IN THE RAT DURING PERIODS OF PHYSIOLOGICAL HYPERTRANSFUSION

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(Ph. D. Thesis)

September 15, 1961

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Contents

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ABSTRACT

A double isotope method for simultaneous measurement of red cell and plasma volumes has been developed for use in the rat. The DFP^{32} red cell life span method has also been adapted to use in the rat, involving test and comparison of three different injection and sampling techniques.

The above measurements, plus Fe^{59} incorporation, were used in various combinations to obtain data on rats in the following physiological states: normal adult male and female rats, hypophysectomized and acutely starved rats, male and female rats reacclimatizing to sea level after a prolonged exposure to simulated high altitude, and male rats exposed to hyperoxia (50% oxygen) for periods up to 1 month. In none of these conditions of "physiological hypertransfusion" was there evidence for a rapid or random destruction or "erythrolysis" in response to the body's decreased needs for erythrocytes.

Male rats reacclimatizing to sea level brought their hematocrits to normal as much by rapid weight gain with a nearly proportional increase in plasma volume as by destruction of red cells. Females, with a less dramatic weight gain, also had a less dramatic decrease in hematocrit. During the reacclimatization process hematocrit and body weight together were excellent predictors of total red cell volume in the rat, and TBH/LVH ratio was quite constant. Thus there was no evidence for redistribution of erythrocytes or a "fluid shift" during reacclimatization to sea level.

Problems associated with measurement of red cell life span in the nonsteady state are discussed, and active destruction defined in terms of an integral equation.

Acute fasting for 4 days caused a 20% decrease in plasma volume and body weight in rats, but no change in red cell volume.

Exposure to 50% oxygen for periods up to 1 month caused a 5% decrease in mean hematocrit and a 10% decrease in red cell volume. DFP^{32} red cell life was normal. Fe^{59} incorporation was briefly depressed, and then returned to control levels.

Red cell volume is studied as a function of the three oxygen tensions at which acclimatized animals were measured--in the normal states, hypoxia, and hyperoxia. Some implications of these findings for oxygen therapy and the control of erythropoiesis are briefly discussed.

I. INTRODUCTION

At times the body finds itself in conditions in which its red cell volume exceeds its current needs. Such states have been referred to as states of "physiological hypertransfusion." They are found, for example, on return to sea level after a sojourn at high altitude, after thyroidectomy or hypophysectomy, or on exposure to hyperoxic conditions. Implicit in the phrase physiological hypertransfusion is the notion that, unlike hypertransfusions of other sorts, they would not be expected to be removed by means of immunologic mechanisms. It is sometimes felt that anotner basic homeostatic mechanism might be identified by investigating the rate of removal of hypertransfusions of an animal's own red cells.

It is well established now that the temporary throttling back of red blood cell production is one of the mechanisms of dealing with hypertransfusion.[1,2] However, there is also a body of evidence interpreted to mean that there may be "active" destruction of red cells as well--that is, that under these circumstances the body is capable of removing cells from the circulation before they finish their normal life span.

The principal observations of this sort have been made on animals or men acclimatized to hypoxia, often at high altitude, who are returned to sea level oxygen pressure.[3-8] Here one has a stimulus that can be applied suddenly and with certainty, whereas after hypophysectomy, for example, the target organ stimuli to erythropoiesis decrease steadily during a period of weeks. A principal effort in this thesis work, therefore, was a quantitative assessment of red cell mass and life span in altitude-acclimatized animals returned suddenly to sea level, in an attempt to remedy what seemed to be deficiencies in previous measurements. The development of the necessary techniques is also discussed in some detail. These techniques made possible further studies that also have a bearing on the way a normal animal handles a rather sudden relative hypertransfusion. These were the measurement of the red cell volume during chronic exposure to high oxygen pressures (Section 6) and after acute fasting (Section 5).

2. BLOOD VOLUME BY SIMULTANEOUS MEASUREMENT WITH IRON-59 RBC AND IODINE-131 ALBUMIN

Introduction

It was considered essential to measure red cell volumes and plasma volumes separately, each with its own indicator. Shifts in fluids between blood and other compartments, or readjustment of the distribution of red cells within the circulation, have been pointed out as factors that might explain the findings in red cell count and hemoglobin when altitude-acclimatized animals are returned to sea level.[3,7] Hence the importance of measuring red cell mass rather than concentration, and of the separate measurement of red cell and plasma volumes. It has been amply shown that the whole-body hematocrit and the large-vessel hematocrit are not the same,[9-15] and that the hematocrit varies widely from one organ to another in rats.[13,16] These findings are discussed in more detail below. It is not yet known to what extent the ratio of whole-body hematocrit to large-vessel hematocrit can vary in different pathological and physiological states[14,15] Hence this ratio was observed during the period after descent from altitude. To reduce the uncertainty in the ratio that is inherent in measuring red cell mass on one group of animals and plasma volume on another, a method was developed for their simultaneous but separate measurement in the same animal.

Fe^{59} has been shown to be an excellent red cell label for blood volumes in small animals.[17] It has tended to be the standard of comparison for other methods, since once incorporated in newly formed erythrocytes it remains for the life of the cell without elution. It also is quite convenient in small animals, and was therefore used in these studies. Iodinated (I^{131}) human albumin has been shown by Everett et al.[16] to be an adequate tagging agent for plasma volume in rats by comparison with C^{14}-labeled rat plasma. The combination of Fe^{59} and I^{131} has desirable characteristics for separate identification of each tracer either by half-life or gamma-ray energy discrimination methods. The separation by half-life was used in these studies. A 6-minute interval

between injection and sampling was arbitrarily chosen for the single sample used in all blood volume measurements, since Berlin et al.[17] have shown that 3 minutes provides adequate mixing time. An effort was made to work out a simple reproducible method for combined blood volumes on rats that could be done with commercially available blood-labeling materials. The method and the various checks on its adequacy are outlined below.

Outline of the Method

The blood volume was determined by simultaneous injection of Fe^{59}-labeled red cells in I^{131} albumin-labeled plasma. Preparation of the injection mixture was begun by intraperitoneal injection of a blood donor rat with 10 microcuries of commercially available Fe^{59} as ferrous citrate from 5 to 14 days prior to the day of blood volume determination. On the day of measurement, 50λ of tail-tip blood was drawn from the donor rat and its counting rate determined in the well-type NaI crystal scintillation counter. An amount of I^{131} albumin calculated to give the same counting rate per unit volume of whole blood as the Fe^{59}--usually about 5 μC--was then injected intravenously into the donor rat under anesthesia. Exactly 6 minutes later, 8 ml of blood was drawn from the aorta of the donor rat into a heparinized syringe. This formed the combined injection mixture. If kept in a stoppered centrifuge tube and handled carefully, it could be used to determine several rat blood volumes during the next 3 to 4 hours.

For blood volume determination 0.2 ml of the donor blood was injected under ether anesthesia into the jugular vein of the experimental animal, and 6 minutes later approximately 8 ml of aortic blood was drawn into a heparinized syringe. A hematocrit was run, and 1 ml each of whole blood and of plasma were counted in the well scintillation counter, along with standards prepared from the donor blood. Background was subtracted from each sample.

Red cell volume was calculated as follows:

$$RCV = \frac{\text{CPM/0.2 ml donor blood minus (1 - Hct donor)} \times \text{CPM/0.2 ml donor plasma}}{\text{CPM/ml recipient blood minus (1 - Hct recipient)} \times \text{CPM/ml recipient plasma}} - 0.2\,\text{ml} \times (\text{Hct donor})$$

$$PV = \frac{\text{CPM/(1 - Hct donor)} \times 0.2\ \text{ml donor plasma}}{\text{CPM/ml recipient plasma}} - 0.2\,\text{ml} \times (1 - \text{Hct donor})$$

The combined blood volume was calculated from the above by

$$BV = RCV + PV .$$

The total-body hematocrit was calculated from the formula

$$TBH = RCV/BV.$$

The ratio of total-body hematocrit to large-vessel hematocrit (TBH/LVH) was obtained by putting TBH as calculated above in the numerator, and the observed hematocrit, with the corrections noted below, in the denominator.

The red cell volumes, plasma volumes, and blood volumes were then also expressed as volume per 100 grams body weight for additional comparisons.

The validity of the above combined method was checked in several ways, outlined below

Checks on Hemolysis and Validity of Double Counting

Any significant degree of hemolysis in either donor or recipient blood while standing or being centrifuged would lead to erroneous results, since the validity of the calculation rests on there being no I^{131} albumin in the red cells, and no Fe^{59} in the plasma at any time before the blood or plasma is in its counting vial.

Hematocrits of donor blood were consistently measured as soon as blood was drawn and again at the end of blood volume measurement. With reasonable handling of the blood, and care to keep the injection syringe perfectly clean and dry, and to keep water or acetone (used in drying) out of the injection mixture, they gave identical results. The

slightest suspicion of hemolysis or clotting led to preparation of a new donor mixture for further determinations that day.

It was then possible to confirm the per cent hemolysis at a later time by waiting 2 months until the I^{131} in all samples had decayed to 0.4% of its initial value, while the Fe^{59} decayed to 40% of its initial value, so that the I^{131} activity was approximately 1% that of the Fe^{59}.

Counts were taken on all samples again at this time, and the per cent hemolysis in donor and recipient animals calculated from the fraction of Fe^{59} present in plasma. It averaged 0.5% and was consistently below 1% hemolysis. The red cell volumes calculated after 60 days' decay of isotopes checked within ±3% with the original determinations. After this delayed check had been found consistent in 75 animals, it was discontinued, and only spot checks on hemolysis were made, beyond observing hematocrit tubes. Thus it was determined that hemolysis did not contribute a significant systematic error to the combined blood volume procedure.

Non-Protein-Bound Iodine-131

In commercial preparations of human I^{131} albumin there are variable amounts of non-protein-bound iodine up to 5% of the total radioactivity. The non-protein-bound iodine might contribute a small and variable systematic error in blood volume determinations. Rather than remove this unbound activity chromatographically or measure and correct for it (an awkward procedure in double counting), it was elected to do a biological separation. Hence the procedure of injecting iodinated albumin into the Fe^{59}-labeled blood-donor rat prior to using its blood. In the donor animal the nonbound iodine was shown to diffuse into a virtual volume seven times as large as the blood volume within 6 minutes, while the bound iodine remained in the plasma volume. A typical figure of 5% nonbound iodine could thus be reduced to 0.8% in the injection mixture. The errors then introduced in the double counting procedure by nonbound I^{131} are all less than 1% and can be neglected for the purpose of these studies.

The nonbound iodine in the commerical preparations was measured by trichloracetic acid precipitation, centrifugation, and radioassay of the supernatant solution. It was shown that TCA precipitation is less than an ideal method; it tends to overestimate the per cent nonbound. But when done after the biological purification of I^{131}-labeled albumin, it established that the nonbound I^{131} was less than 1% of the total I^{131}. TCA precipitation at near freezing temperatures consistently gave slightly lower figures for per cent nonbound than did room-temperature determinations. Using TCA precipitation on material measured directly and after biological purification, combined with the figure for the 6-minute iodide space of the rat (see below), a calculation indicated that TCA precipitation itself gave rise to a figure for the nonbound fraction which was spuriously high by 1.8%. Correction for this effect could then be made. Obviously chromatographic separation of the iodine, either before administration or for measurement, might give neater data, but the accuracy of the blood volume method was adequately established with TCA precipitation. In addition, this effect of TCA precipitation on the iodine bound to protein is of some interest.

The 6-Minute Iodide Space of the Rat

To ascertain the relative extent of dilution of iodide over iodinated albumin in the rat, five rats were injected with 0.2 ml each of solution containing I^{131} as iodide. The mean value for "iodide space" at 6 minutes after injection was 35.6 ml/100 g rat. The mean value for iodide activity per milliliter of red cells was 0.71 times that per milliliter of plasma, and the virtual volume of dilution for iodide at 6 minutes (calculated from its mean concentration in whole blood) is 40.9 ml/100 g. Since the mean blood volume is 5.9 ml/100g, the "purification factor" on putting the commerical material through the donor rat is nearly seven.

Hematocrit Plasma-Trapping Correction and Centrifuging Methods

Numerous authors have found that when hematocrits are measured by ordinary centrifugation, plasma is trapped in the red cell mass, causing a systematic error known to vary with speed and time of centrifugation.[18, 19] Owen reviewed these measurements and, using I^{131} albumin in an "indirect" method (in which whole blood and plasma are counted), he found that the true hematocrit is 4% lower than the observed hematocrit in humans.[19] Since this is a systematic error that can be allowed for, it was desirable to apply it to the rat studies. So that the correction would be directly applicable to these studies, it was determined in Long-Evans rats, using our own conditions of centrifugation, and with a wide range of hematocrits.

Normal rat red cells were made up in heparinized whole rat plasma to have hematocrits from seventeen to seventy. Ten determinations were then made with I^{131} albumin by the indirect method counting 1 ml whole blood and 1 ml plasma in the well counter. The "true hematocrit" $= 1 - \frac{\text{CPM in 1 ml whole blood}}{\text{CPM in 1 ml plasma}}$.

The "observed hematocrit" was obtained by centrifuging blood in a Wintrobe tube for 1 hour at 2500 rpm in a standard large laboratory centrifuge at room temperature. Observed hematocrit values were routinely rechecked after 2 hours, also, as an additional safeguard against errors, but the 1-hour value was used in computation. In the ten normal blood samples of varying hematocrit, it was found that the true hematocrit was 0.955 ± 0.022 times the observed hematocrit, and the ratio did not change appreciably with change in hematocrit. Since this value is consistent with the well-established systematic error of 0.96 for such determinations in humans,[20] all observed hematocrit values were multiplied by 0.955 to obtain the "true" hematocrits recorded in this report.

Variation in Hematocrit with the Sampling Method

Since the total-body hematocrit is smaller than the large-vessel hematocrit by 15 to 18%,[9-15] the amount of blood sampled in a small animal should influence the hematocrit measured. An estimate was made of this effect in three rats. The blood was obtained by sampling from the dorsal aorta at its bifurcation. The first 3 ml of blood was obtained within 1 to 2 minutes in separate 1-ml amounts. The remaining 3 to 5 ml was obtained in a single syringe in an additional 1 minute's time. Hematocrits were measured on all samples in the way previously outlined.

The data indicated that the mean hematocrit of the first eight milliliters is 96% of that of the first or third milliliter obtained. Since this is a systematic finding, one might correct all hematocrits obtained in sampling 8 ml, so that TBH/LVH ratios would be expressed as the TBH compared with a very small sample of aortic blood. However, in this study, large samples were required, and the LVH discussed in other sections is arbitrarily defined as the hematocrit actually measured on 8 ml aortic blood multiplied by the plasma-trapping correction. It is the same as the "true hematocrit" discussed above.

It is interesting that approximately one-half of the rat's blood can be drawn from its aorta while its heart is still beating, and that the hematocrit of this sample is closer to that of a small sample of aortic blood than to its total-body hematocrit Indeed, the first 20% of its blood volume withdrawn in this way will have a hematocrit identical with that of a small aortic sample.

3. DFP^{32} RBC LIFE SPAN

Introduction

Di-isopropyl fluorophosphate tagged with P^{32} (DFP^{32}) combines irreversibly with a wide variety of proteins. It has now been used successfully by a number of workers to label blood constituents.[20-23] It is a particularly attractive agent for red cell life span studies: given intravenously it labels red cells of all ages, and remains firmly bound to them throughout their life span, so that its disappearance from red cells follows the typical Ashby curve.[24-27] Reutilization of DFP does not occur. Initially it also labels plasma proteins (as well as numerous proteins outside the circulation) so that red cell and plasma activity must be separated for the first few days, until the more rapid plasma turnover has reduced plasma labeling far below the red cells. One can measure directly the activity of washed red cells. An alternative method is to measure whole blood and plasma and calculate the red cell activity indirectly. We checked the two methods and showed their results to be essentially identical, and then proceeded with the indirect method. The relative numbers of white cells and platelets are so small, and their labeling not exceptionally high, that they can be neglected in the indirect method.

DFP^{32} was used to measure red cell life span during the period after descent from simulated high altitude. These results are given in detail in Section 4. It was also used to measure red cell life span on exposure of animals to increased ambient oxygen (Section 6). In the development of sampling techniques, DFP^{32} was also used in some additional studies on normal and hypophysectomized rats. Results of these latter experiments are outlined in this section.

Outline of Methods

Owing to the low specific activity of the available DFP^{32}, and the low counting rates toward the end of the experimental period, an initial experiment was done to determine a tolerable dose of DFP carrier in the rat. This is a highly toxic substance, yet it was desirable to work near the tolerable limit.

Rapid intravenous injection of the vehicle, propylene glycol, alone, was first tried. It was tolerated in amounts up to 2.3 ml/kg without observable effect during the first week thereafter. Carrier (non-radioactive) DFP in propylene glycol iv in amounts up to 0.8 mg/kg caused no observable effect, and up to 2.3 mg/kg caused no acute lethality. Between 0.8 and 2.3 mg/kg, excessive salivation and tetanic convulsions appeared in increasing amount. Animals that survived the first hour after injection in this dose range were hyperactive and showed some posterior extremity weakness for 1 to 7 days, but progressively returned to normal. As will be seen below, doses one-fourth to one-half of those producing observable effects were used successfully for the red cell life span studies. This powerful anticholinergic drug has been used extensively now for cell life span studies in humans, at doses around the threshold of symptoms. There are no untoward long-term symptoms known at such doses.[20, 21, 28]

Early experiments indicated that tail vein injection of the material was not reliable because of occasional extravascular leakage at the injection site; also pericardial puncture for sampling was inadequate owing to formation of sizeable hemopericardia. Some preliminary work with Contopolous* led to the use of the technique of iv injection in the jugular vein under ether anesthesia, a method permitting accurate and reproducible iv dose administration and a minimum of blood loss prior to sampling.

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*Dr. Alex Contopolous of the Institute of Experimental Biology, University of California, Berkeley.

Uncertainty as to stability of the material in saline dilutions led us first to make up the dilutions immediately before use, and later to administration of the DFP intravenously in pure propylene glycol. Hence the testing of tolerance to intravenous propylene glycol described above. One-fourth-milliliter specimens of whole blood were drawn from alternate groups of animals at 3-day intervals so that each animal was sampled only every 6 days. However, this was a sizeable blood loss even in normal animals, and especially so in hypophysectomized rats or those in which one is attempting to study a changing red cell mass. There was also some visible hemolysis in these samples. Thus other sampling techniques were developed (see below). This preliminary work also indicated that the indirect method of counting whole blood and plasma was just as accurate as measuring red cells washed in rat plasma. The work on return to sea level after altitude acclimatization (Section 4) was done with jugular vein injection of approximately 20 μC DFP^{32} with 0.1 mg carrier DFP in 0.1 ml propylene glycol, and sacrifice of groups of animals at intervals to obtain aortic blood samples. One-milliliter whole blood and 1 ml plasma were then separately plated and counted on a wrap-around Geiger tube. After decay and other counting corrections had been applied, the counting rate per milliliter of RBC and of plasma were separately plotted against time.

In a later experiment to determine whether individual rats could be followed with repeated small samples without significant disturbance to erythropoietic balance, DFP was given ip to each of six rats, and frequent tail-tip samples amounting to less than 100λ were used to obtain microhematocrit and counting rate of whole blood and plasma. The results obtained by this method agreed with the value for normal red cell life span in the rat obtained by the method of iv injection and sacrificing many animals. It was therefore used also in the final experiment on increased ambient oxygen (Section 6).

Results

Preliminary work (done in cooperation with Dr. Alex Contopolous) indicated that red cell and plasma labeling with DFP^{32} were about equal in amount, and that the plasma labeling thereafter decreased exponentially with a half time of 2 days. (Fig. 1). The red cells had a rather high amount of labeling at 2 days, with wide variation between animals, followed by a much less variable degree of labeling with a linear decrease after 4 days. Van Putten found this same effect in even greater amount and discussed it in terms of a labile as well as a stable red cell tagging.[27] Our experiment indicates that this "labile" tag on red cells cannot be removed by repeated washing with rat plasma. The cause of this labile tagging is unknown at present.

The life span of the red cell in the hypophysectomized rat was 46 days, not significantly different from the normal rat (see below). This is in agreement with the result of C^{14}-glycine labeling by Berlin et al.[29]

By using the intravenous injection and aortic sampling, in controls and animals returned from altitude, again the plasma was found to label about 1-1/2 times as much as the cells at 2 days, and to lose its label with a half time slightly under 2 days. No labile tagging of red cells was noted on this occasion. The 2-day point for red cells fell on the straight line of the Ashby curve with the later red cell values in both control and experimental groups. The red cell life span for normal LE male rats was 48 days.

In the next experimental group, in which individual normal animals were repeatedly sampled, the variable initial labeling was again seen in about half the animals. The mean red cell life span in this group of normal LE male rats was 38 days (Fig. 2).

The final DFP experiment under increased ambient oxygen again showed the labile red cell labeling.

More detail on the DFP^{32} labeling of cells after descent from altitude and exposure to increased oxygen is given in Sections 4 and 6.

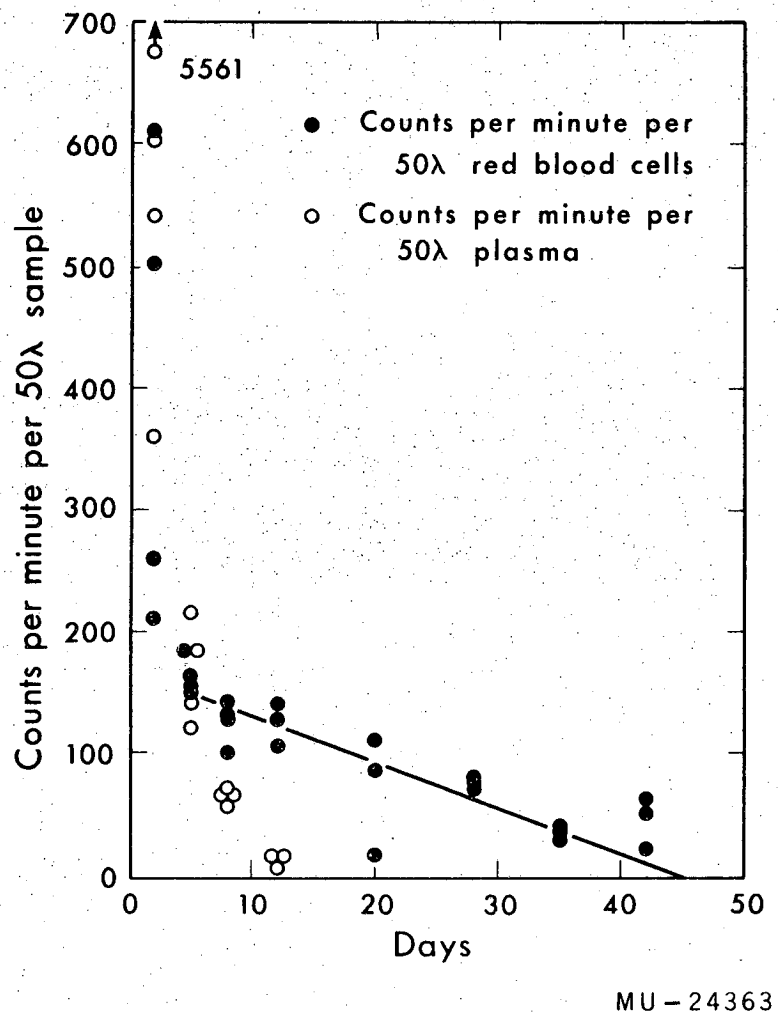

Fig. 1. DFP^{32} red cell life span in the hypophysectomized rat. Least-squares straight line fitted to red cell activity from 4 to 35 days intersects the base line at the mean red cell life span.

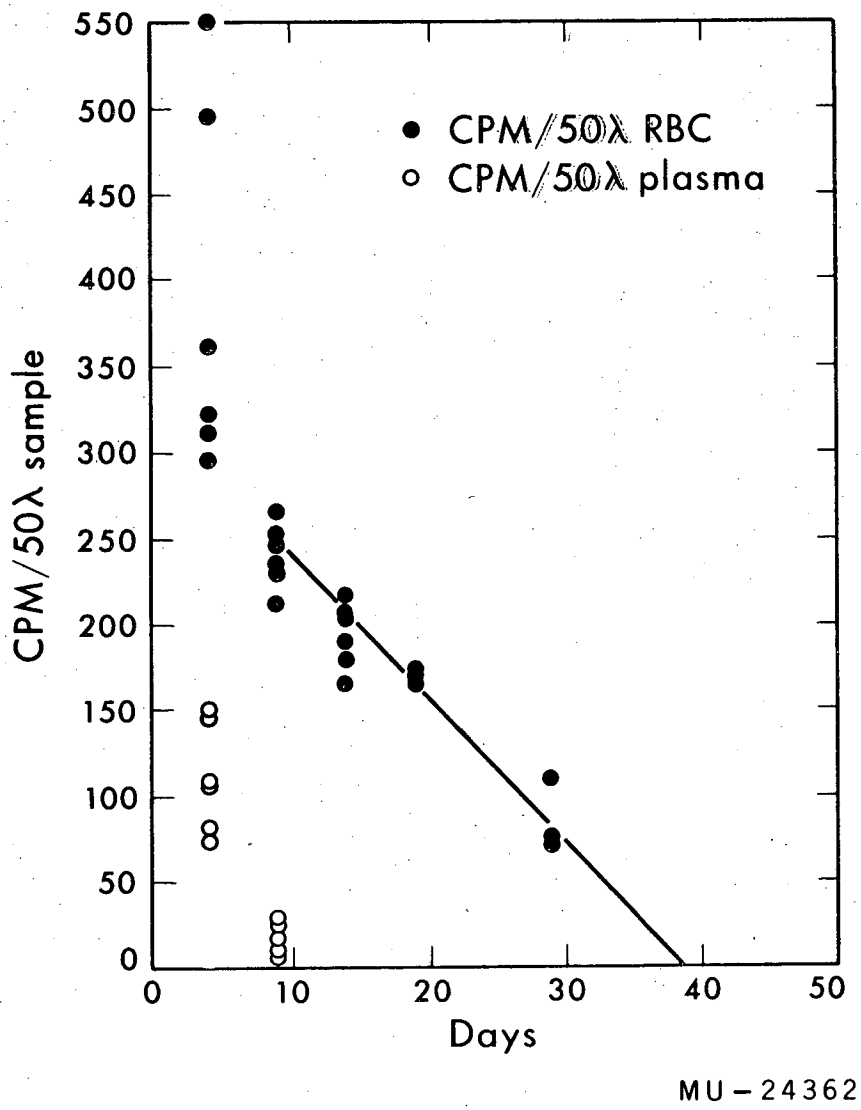

Fig. 2. DFP^{32} red cell life span in the normal male LE rat, repeated small samples.

Discussion

Using C^{14} glycine, Berlin found the red cell life span of Curtis-Dunning rats to be 60 to 68 days.[30] Van Putten[27] reviewed this finding, and the methods of Burwell and co-workers, which gave a life span of 45 to 50 days in rats (strain not given) with Fe^{59} and iron loading. While this manuscript was in preparation, Van Putten published the first data on the life span of red cells in the rat and mouse as determined by labeling with DFP^{32} in vivo.[27] In male W. A. G. rats he obtained linear Ashby curves giving a red cell life span of 60 days. Our result was 38 to 48 days in Long-Evans males. Strain differences may explain the discrepancy. Differences in resistance to the ubiquitous Bartonellosis of ordinary laboratory strains of rats cannot be ruled out, either.

Berlin has shown that the red cell life span is normal in hypophysectomized rats, using C^{14} glycine.[29] The results obtained here confirm his finding using DFP^{32}. The life span obtained was 46 days.

4. RED CELL MASS AND LIFE SPAN ON RETURN OF ALTITUDE-ACCLIMATIZED RATS TO SEA LEVEL

Introduction

Evidence for active red cell destruction or "erythrolysis" on return of altitude-acclimatized men and experimental animals to sea level has been ably reviewed by Pace et al. (3). The various methods used to asses active destruction by Fryers and Berlin, Merino, Reissmann et al., Pace et al., Reynafarje, and others have yielded a mass of conflicting data (1-8). One of the problems is a careful definition of "active destruction" or "erythrolysis." Active destruction implies that in the normal animal the life span of red cells is not just a function of their age, but can be altered by other factors to a significant degree in response to changes in the body's needs. Passive removal of cells implies that only their age affects their rate of removal from the circulation. A careful application of this definition requires complicated curve fitting depending on the previous history of red cell production. This is presented in more detail in the discussion at the end of this section. However, our data, as well as those so far gathered by others, seem only to warrant the kind of mathematical treatment usually used--one requiring the assumption that the animal has been long enough at altitude that his red cell production has leveled off, and that the age distribution of red cells is comparable to the sea-level age distribution. In this case, passive removal of cells, on descent from altitude should give a simple linear decrease in cells formed prior to descent, and at a rate consistent with the usual sea-level or altitude life span.

Difficulties with experimental techniques offer further complications that may account for the conflicting results noted above. Several of the methods used have been treated as if quantitatively meaningful, when they have subsequently been shown or can be shown to be only semiquantitative. Among these are radioactive iron uptake in its simpler forms,[31] reticulocyte percentage,[32] and the urobilinogen excretion and index.[6] Some of these measurements tend to give the right answer in the steady state, but not necessarily when conditions

are changing. The urobilinogen excretion has the further disadvantage that urobilinogen has now been clearly shown not to be derived entirely from red blood cells.[2, 33] The good agreement which has appeared at times in urobilinogen excretion results may be falsely reassuring.

Measurement of red cell count, hematocrit, or hemoglobin might give a false impression of what is happening to red cell mass, owing to fluid shifts or redistribution of blood, as Pace et al. pointed out.[3] They measured only hematocrit and red cell count in mountaineers descending from altitude, and showed that unless there were significant fluid shifts their data were compatible only with active destruction of red cells after descent. Reissman et al. obtained the same sort of result in dogs.[4] They measured red cell volume with a plasma-labeling agent, because infusion of tagged cells would have interfered with their hemoglobin catabolism measurements. Reissmann et al.[4] and Pace et al.[3] first clearly pointed out that measurement of the rate of decrease of total red cell mass may at times constitute a crucial experiment: if the red cell mass actually falls faster than can be explained by a total cessation of production plus a normal life span, there must be active destruction. It is also possible that very active destruction is going on undetected, if production of new cells is also going on at a rate close to its usual value. In that case the combination of measurements of red cell mass and life span presented in Experiment II below would be required to show the effect. The research outlined below in the first experiment of this section was an effort to measure this rate of change of red cell mass with a red cell label, which had not been previously done, and in addition to simultaneously measure plasma volume with a plasma label so that information could be obtained on fluid shifts or redistribution of red cells, as a possible cause for the changes in hematocrit.

A thoroughgoing test of the active vs passive hypotheses for red cell life span during reacclimatization to sea level would consist of quantitative measurements of rate of red cell formation, total circulating red cell mass and its rate of change, and red cell destruction

rate, all three simultaneously. Such a study would differentiate between the two hypotheses and also demonstrate that the three measurements balance out against one another. However, as mentioned above, the present methods for measurement of red cell production in the rat are only semiquantitative. This statement applies to Fe^{59} red cell uptake as well as to marrow cellularity or reticulocyte count. Thus the actual tests of the active destruction hypothesis have been less exhaustive, and the complete balance from the three measurements has not been achieved.

Using C^{14} glycine in rats, Fryers and Berlin measured red cell life span of a population of red cells formed just before descent from altitude.[34] They obtained a normal life span (consistent with passive destruction). They did not measure the changing red cell volume during this period, however. Thus they obtained only specific activity rather than total activity. There was the possibility that either random destruction or destruction only of old cells would go unrecognized in such a system. A correction for changing red cell mass was desirable. Also, as Pace et al. pointed out, it was desirable to recheck the red cell life span with an agent that labeled cells of all ages.

Recently Reynafarje reported on the measurement of both C^{14} glycine and Cr^{51} red cell life spans in residents at high altitude taken to sea level.[1,2] They were within normal limits. He gives an excellent discussion of some of the problems with bile pigment excretion, and suggests, as we have felt, that Merino's finding of increased destruction rate on the basis of increased bilirubin excretion[6] may not be valid, and that the red cell life span methods are more straightforward. Unfortunately Reynafarje does not comment on how often red cell volume was measured after descent, or specifically whether the correction for changing blood volume was applied.

Thus the combination of the DFP^{32} life span and red cell volume measurement repeated on the rat returned to sea level after altitude acclimatization seemed to be a straightforward approach to a definite answer about active destruction, and to clearing up some of the conflicting results.

The measurements fell into three principal experiments. Each of these has its own problems and advantages for answering the question, "Is there active destruction of red blood cells during this period when red cell mass is returning to normal?" The experiments were

(I) frequent measurements of red cell mass and plasma volume during the postaltitude period. This was done in male LE rats exposed to 15,000 feet for 30 days.

(II) DFP^{32} labeling of red cells during the same period, also on male LE rats exposed to 15,000 feet for 30 days.

(III) A repeat of experiment I in female LE rats exposed to 18,000 feet for 45 days.

The inferences drawn from combination and comparison of these three experiments are given below.

Experiment I. Blood volume of Male Rats After 30 Days at 15,000 Feet

The initial experiment began with the separation of 75 male LE rats weighing 200 to 250 grams into control and experimental groups at random. The experimental animals were then kept in the Donner Laboratory altitude chamber at a simulated altitude of 15,000 feet for 30 days. A period of 20 minutes daily of sea-level pressure was permitted in order to simplify animal care. At the end of the 30 days, red cell volumes and plasma volumes were measured in control and experimental animals with the combined Fe^{59} RBC-I^{131}-albumin technique described in Section 2. This was done at intervals of 1 to 3 days at the beginning, decreasing in frequency toward the end of the first 20 days after return to sea level, when the control level of red cell volume had been reached.

Table I presents the data obtained. Initial examination of the hematocrit and of the red cell volume per 100 grams (Fig. 3) made it appear that there was a dramatically rapid rate of decrease of red cell mass compatible only with active destruction of red cells--that is, a line of decrease falling significantly below the line for a 45-day finite life span.

Table I. Red cell values in adult male LE rats after removal from 30 days in altitude chamber at a simulated altitude of 15,000 feet.

No. of days	RCV	PV	BV	TBH	LVH	TBH/LVH	Weight (g)	No.	RCV/100 g	PV/100 g	BV/100 g
Control 0	6.2 ± 0.3	11.5 ± 0.6	17.8 ± 0.5	35.0 ± 0.6	40.0 ± 0.9	.87 ± .01	301 ± 12	6	2.06 ± .03	3.82 ± .15	5.88 ± .08
Expt. 0	8.7 ± 0.3	8.0 ± 0.3	16.7 ± 0.5	52.1 ± 0.7	58.6 ± 1.3	.89 ± .01	234 ± 7	7	3.72 ± .10	3.43 ± .09	7.15 ± .16
Expt. 0.6	8.8 ± 0.8	8.8 ± 0.3	17.5 ± 0.3	49.7 ± 2.0	57.6 ± 3.0	.87 ± .06	242 ± 12	5	3.61 ± .20	3.63 ± .11	7.25 ± .19
Expt. 1.6	8.5 ± 0.4	9.5 ± 0.6	18.0 ± 1.0	47.4 ± 1.4	55.8 ± 1.1	.85 ± .03	246 ± 11	5	3.48 ± .12	3.86 ± .13	7.34 ± .14
Expt. 2.6	7.1 ± 0.2	9.5 ± 0.5	16.6 ± 0.7	42.7 ± 1.0	51.8 ± 0.9	.82 ± .02	225 ± 2	3	3.14 ± .08	4.23 ± .20	7.37 ± .25
Expt. 3.6	7.6 ± 0.2	9.5 ± 0.5	17.1 ± 0.8	44.6 ± 0.9	53.4 ± 0.4	.83 ± .02	237 ± 6	3	3.22 ± .30	4.00 ± .19	7.22 ± .34
Expt. 7.6	6.8 ± 0.3	9.7 ± 0.2	16.5 ± 0.1	41.4 ± 1.4	48.2 ± 1.7	.86 ± .04	255 ± 4	5	2.68 ± .07	3.81 ± .12	6.49 ± .08
Expt. 11.6	7.0 ± 0.4	11.6 ± 0.5	18.6 ± 0.9	37.2 ± 1.1	45.2 ± 0.9	.82 ± .03	275 ± 12	5	2.52 ± .08	4.24 ± .12	6.76 ± .13
Expt. 14.6	7.5 ± 0.2	11.8 ± 0.7	19.3 ± 0.7	39.2 ± 2.0	45.1 ± 2.7	.87 ± .07	299 ± 5	5	2.52 ± .07	3.95 ± .22	6.47 ± .25
Expt. 19.6	7.4 ± 0.4	12.6 ± 0.8	20.0 ± 1.1	36.9 ± 1.5	45.5 ± 2.2	.81 ± .05	318 ± 21	4	2.33 ± .09	3.97 ± .12	6.30 ± .11
Control 11.6	7.1 ± 0.1	12.3 ± 0.5	19.4 ± 0.7	36.7 ± 0.6	43.0 ± 0.8	.85 ± .02	318 ± 5	3	2.24 ± .06	3.87 ± .24	6.12 ± .31
Control 19.6	7.2 ± 0.1	13.3 ± 0.3	20.6 ± 0.2	35.2 ± 0.9	42.0 ± 0.5	.84 ± .03	334 ± 6	4	2.16 ± .06	3.99 ± .10	6.16 ± .11
All Control	6.73 ± .19	12.27 ± .37	19.0 ± .52	35.5 ± 0.5	41.4 ± 0.5	.86 ± .01	315 ± 7	13	2.14 ± .03	3.88 ± .07	6.02 ± .09

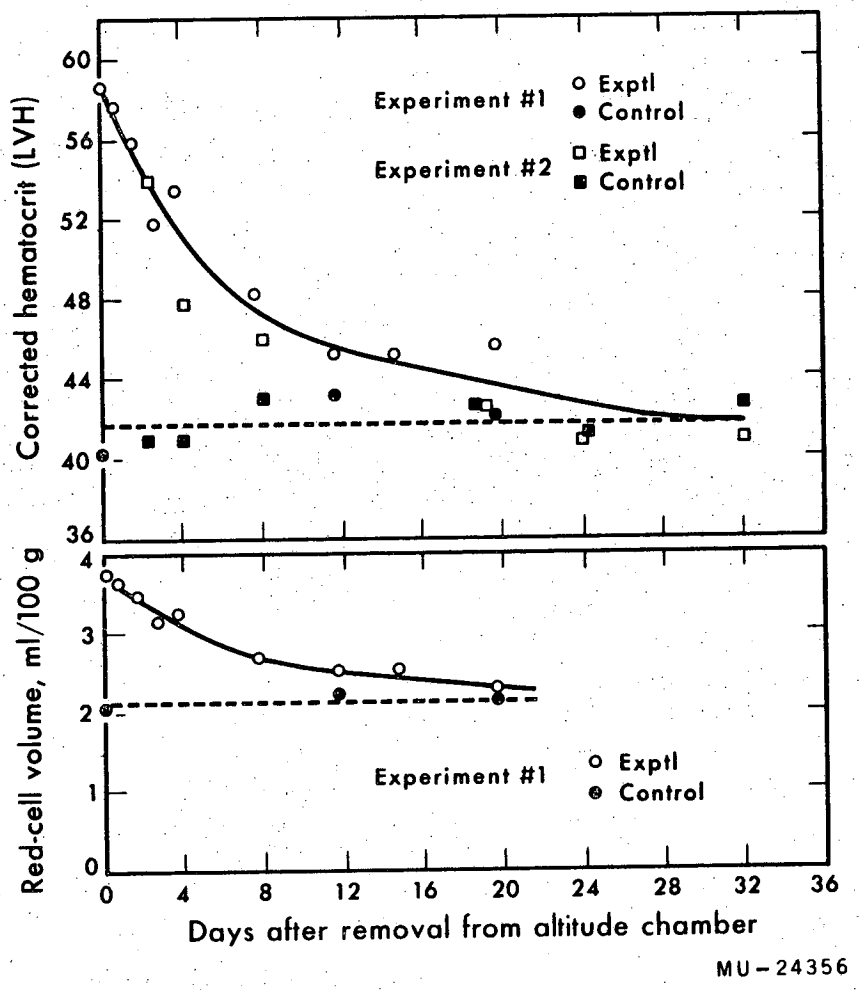

Fig. 3. Mean hematocrit and red cell volume per 100 g in male LE rats after removal from 30 days in the altitude chamber (15,000 feet).

However, the experimental animals undergo a rapid weight gain with a proportionate increase in plasma volume after return from altitude (Fig. 4). Therefore, the rate of decrease of the total red cell volume was considerably less (Fig. 5). At its steepest it fell along a regression line suggesting a 31-day life span rather than 45 days. It was not different from the 45-day line with a significance greater than $p \sim 0.1$ to 0.15. Also, the curve for total red cell volume came lowest at about 8 days, when it had decreased only 16%, and then began to rise again because of rapid weight gain and the requirement for a larger red cell mass on that basis. Thus the male rate compensates the relative hypertransfusion as much by growth as by destruction of red cells.

The effort to reduce variability by plotting red cell volume per 100 g weight actually decreased standard errors only slightly, and seemed to have led us to an initial interpretation as confusing and possibly spurious as measurement of red cell counts or hematocrit only.

In some human descents from altitude on high-mountain expeditions there may also be this same increase in weight. The effect could presumably be avoided to some extent by exposure of larger animals under the less stressful conditions of the altitude chamber. Committed to working through the details of this phenomenon for rats, we repeated it, using female rats, whose rate of weight gain during adult life is considerably less than that of males (see Experiment III).

Experiment II. DFP^{32} Labeling of RBC in Male Rats After 30 Days at 15,000 Feet

The animals were prepared as in Experiment I. Fifty-seven male LE rats of 200 to 250 g were used, half of which were kept at 15,000 feet for 30 days. On the day of descent from altitude each experimental and control animal was injected with approximately 20 μC of DFP^{32} plus 0.1 mg carrier DFP in 0.1 ml propylene glycol. The sampling method outlined in Section 3 was followed, sacrificing groups of animals at intervals for aortic blood samples. Thus

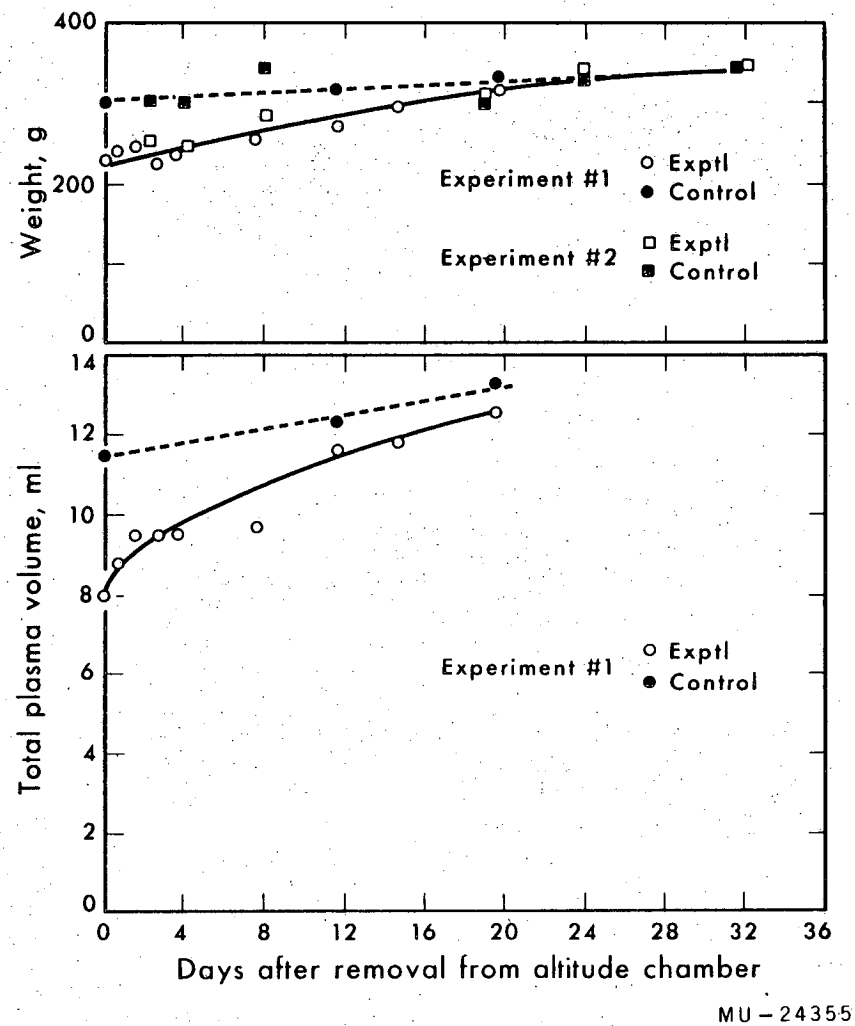

Fig. 4. Mean weight and total plasma volume in male LE rats after removal from 30 days in the altitude chamber (15,000 feet).

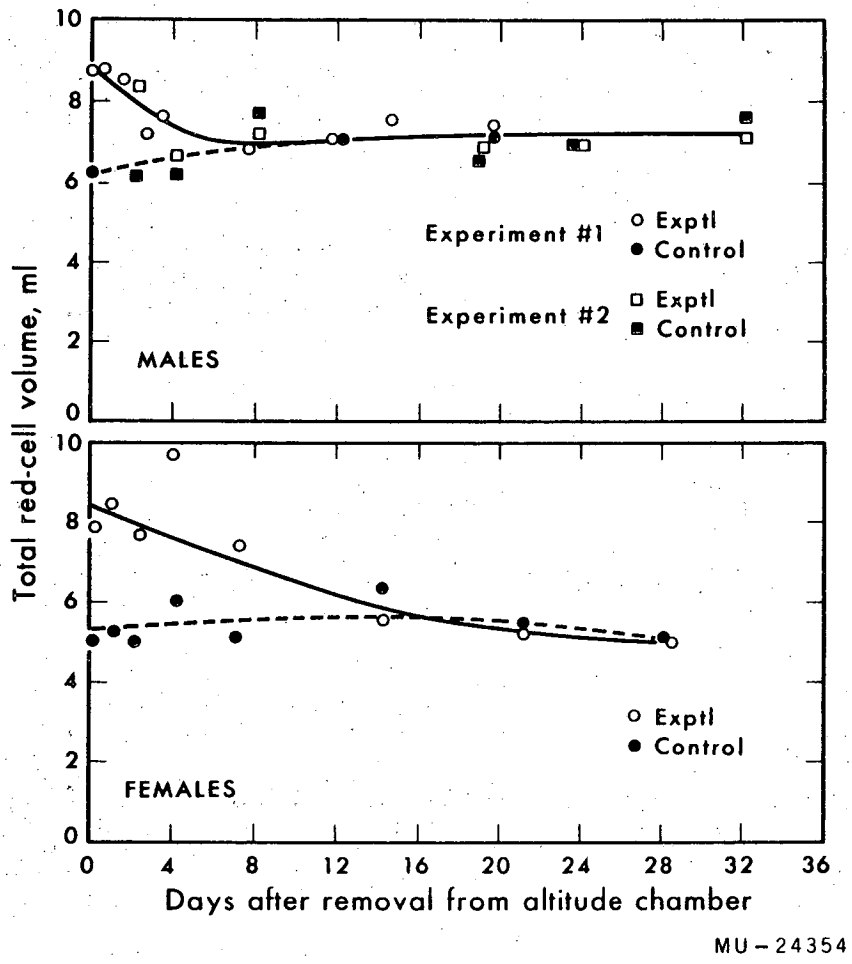

Fig. 5. Total red cell volume (group means) in adult LE rats removed from the altitude chamber (males 30 days at 15,000 feet, females 48 days at 18,000 feet).

preparation and sampling of these rats were closely comparable to that in Experiment I above.

The results of these measurements are summarized in Fig. 6. The uncorrected specific-activity data for the experimental group (not shown) actually extrapolates to a considerably longer life span for the altitude than the sea-level group. However, the most significant test is the rate of decrease of total labeled red cell mass, i.e. the specific activity multiplied by the red cell mass. Thus the data of Experiments I and II can be combined to show the rate of destruction of the red cells of all ages which were labeled on the first day after descent from altitude. The corrected curve (Fig. 6) is reasonably linear. Its slope is not significantly different from the control, extrapolating to a life span of 66 days, while the control was 55 days.

The difference between the 55-day control value here and the 45 days in other parts of this report is partly because it was necessary here to make a correction for weight gain in both altitude and control animals. Neglect of the effect of weight gain on total labeled activity--that is, calculation from specific activity of blood alone--leads to a calculated 48-day life span in the controls. There is thus a systematic underestimate of life span of the same magnitude in the DFP^{32} red cell life spans given in Section 3, but the values there are directly comparable to those for small animals given by most authors, who also do not correct for weight gain.

The animals of Experiments I and II were quite comparable in the time course of their weight gain and hematocrit change, in addition to the fact that experimental conditions were kept as nearly identical as possible. Therefore, it proved immaterial whether the correction for changing red cell volume was done from a freehand curve, or the multiple regression of red cell mass on hematocrit and weight as a basis for combining the experiments. The latter was used in Fig. 6.

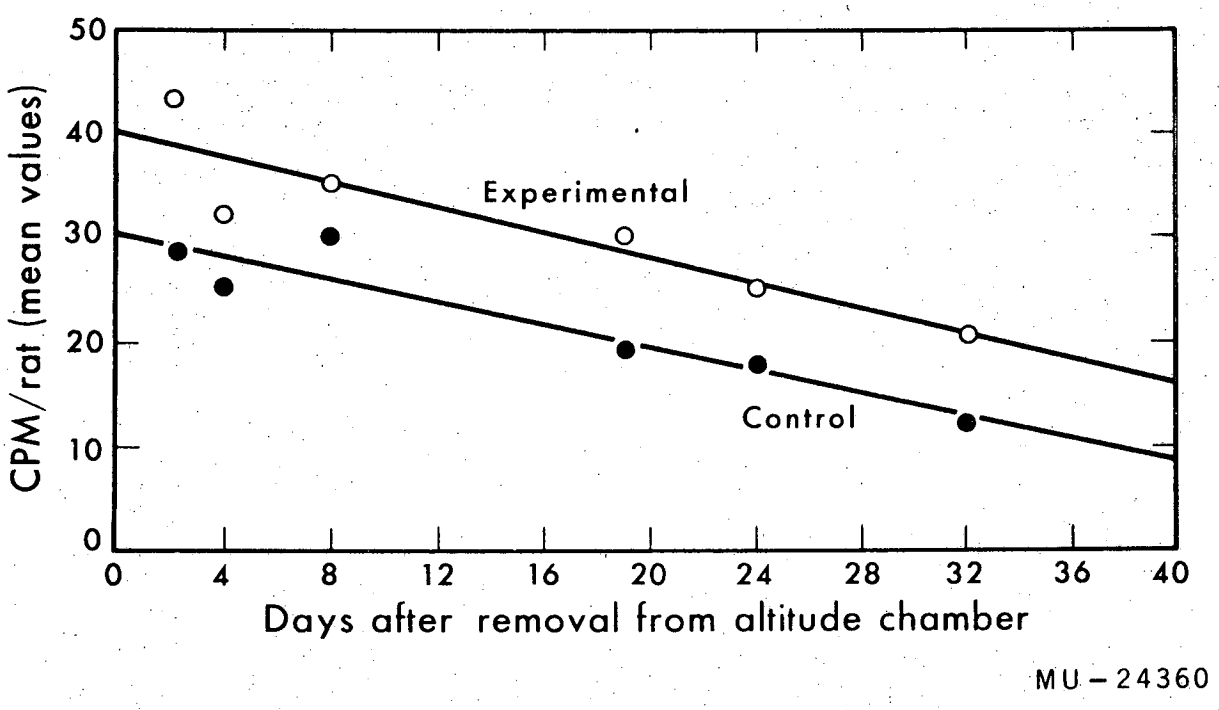

Fig. 6. Total DFP^{32} activity per animal (group means) plotted against time after removal from altitude chamber. Adult LE male rats of Experiment II with red cell volume correction from Experiment I. Least-squares linear fit to data from days 2 through 32.

In larger animals, of course, all these measurements can be combined in the same animal, though fewer can be tested. Recently Van Dyke made such measurements in a monkey and failed to find evidence for active red cell destruction.[35]

To establish whether the 66-day life span was really different from the 55-day controls would require a great many more experiments.

Experiment III. Blood Volume of Female Rats After 45 Days at 18,000 Feet

In repeating Experiment I to make another independent assessment of the "active destruction" thesis, some changes were made in hopes of bringing out any such effect more dramatically. Seventy-five female LE rats were used, to see if the thesis applied also to females, but primarily to reduce the effect of weight gain during the experimental period. They were exposed to 18,000 feet rather than 15,000, to increase the polycythemia, and they were kept there 45 rather than 30 days. In other respects these animals were handled as in Experiment I. The data obtained are shown in Table II. The results are generally comparable to those for males. However, in this case neither red cell mass nor red cell mass per 100 g decreased at a rate significantly different from a 45-day life span.

Discussion

As mentioned above, plasma volume increased almost in proportion to weight gain, so that the plasma volume per 100 g weight appeared to increase only slightly in males and not at all in females. This indicates that, at least in the rat, hemodilution (in the ordinary sense) is not a significant feature in return from altitude. Whole blood volume per 100 g decreased slightly in both males and females. Because of the weight gain this came about in the face of an actual slight increase in total blood volume during the experimental period for the males.

One finding in both sexes was a quite constant ratio of total-body hematocrit to large-vessel hematocrit (TBH/LVH in Tables I and II). In normals, in animals acclimatized to high altitude, and even

Table II. Red cell values in adult female LE rats after removal from 48 days in altitude chamber at a simulated altitude of 18,000 feet.

No of days	RCV	PV	BV	TBH	LVH	TBH/LVH	Weight (g)	No.	RCV/100 g	PV/100 g	BV/100 g
Control 0.2	5.1 ± .1	9.6 ± .5	14.7 ± .6	34.6 ± 0.5	43.5 ± 1.0	.80 ± .01	260 ± 7	3	1.96 ± .06	3.70 ± .18	5.66 ± .23
Expt. 0.2	7.9 ± .6	8.0 ± .2	15.9 ± .8	49.3 ± 1.5	59.6 ± 1.2	.83 ± .01	235 ± 6	9	3.34 ± .18	3.40 ± .09	6.74 ± .20
Control 1.2	5.3 ± .1	8.5 ± .1	13.8 ± .1	38.2 ± 0.5	45.5 ± 0.5	.84 ± .02	241 ± 5	3	2.18 ± .05	3.54 ± .11	5.72 ± .11
Expt. 1.2	8.5 ± .9	8.3 ± .3	16.8 ± 1.2	50.3 ± 2.0	56.8 ± 4.0	.90 ± .05	242 ± 9	4	3.48 ± .28	3.41 ± .01	6.89 ± .28
Control 2.2	5.0 ± .1	9.4 ± .3	14.5 ± .3	34.8 ± 0.9	43.1 ± 1.6	.81 ± .01	254 ± 6	2	1.98 ± .04	3.73 ± .21	5.71 ± .25
Expt. 2.2	7.7 ± .6	8.5 ± .1	16.2 ± .5	47.3 ± 1.9	55.8 ± 2.1	.85 ± .01	225 ± 4	4	3.41 ± .28	3.77 ± .04	7.18 ± .30
Control 4.2	6.1 ± .4	9.4 ± .4	15.4 ± .7	39.3 ± 0.5	41.8 ± 0.7	.94 ± .01	265 ± 2	2	2.29 ± .15	3.54 ± .16	5.83 ± .31
Expt. 4.2	9.7 ± .8	7.0 ± .3	16.7 ± .9	57.4 ± 2.3	64.4 ± 2.5	.89 ± .02	232 ± 5	8	4.16 ± .31	3.03 ± .12	7.19 ± .30
Control 7.2	5.2 ± .2	10.5 ± .4	15.7 ± .5	33.0 ± 0.7	42.6 ± 1.3	.78 ± .02	283 ± 19	3	1.84 ± .08	3.73 ± .16	5.57 ± .21
Expt. 7.2	7.4 ± .4	9.0 ± .4	16.5 ± .6	45.1 ± 2.0	53.6 ± 1.9	.84 ± .01	246 ± 12	5	3.04 ± .20	3.68 ± .10	6.73 ± .18
Control 14.2	6.4 ± .7	11.3 ± .6	17.7 ± 1.4	36.1 ± 1.4	47.7 ± 1.4	.76 ± .01	302 ± 13	2	2.12 ± .15	3.74 ± .04	5.86 ± .20
Expt. 14.2	5.6 ± .2	9.8 ± .3	15.4 ± .2	36.3 ± 1.3	49.4 ± 2.4	.74 ± .01	260 ± 10	5	2.16 ± .10	3.79 ± .04	5.95 ± .15
Control 21.2	5.5 ± .5	9.3 ± .7	14.8 ± 1.1	37.3 ± 2.0	42.5 ± 2.1	.88 ± .01	262 ± 15	3	2.10 ± .16	3.54 ± .17	5.64 ± .13
Expt. 21.2	5.3 ± .2	9.5 ± .8	14.8 ± 1.0	36.1 ± 1.6	40.9 ± 1.1	.88 ± .03	249 ± 15	5	2.13 ± .16	3.79 ± .17	5.95 ± .18
Control 28.2	5.2 ± .5	10.2 ± .7	15.4 ± .9	33.5 ± 2.5	42.1 ± 1.3	.79 ± .04	259 ± 12	4	1.99 ± .12	3.97 ± .26	5.96 ± .22
Expt. 28.2	5.1 ± .3	9.4 ± .4	14.5 ± .7	35.3 ± 0.5	41.3 ± 1.0	.86 ± .01	268 ± 14	5	1.91 ± .01	3.50 ± .10	5.41 ± .12
All Control	5.39 ± .14	9.77 ± .21	15.2 ± .3	35.6 ± 0.6	43.4 ± .01	.82 ± .01	264 ± 5	22	2.04 ± .04	3.70 ± .07	5.74 ± .21

during the first days after return to sea level there was no change in ratio, the average remaining 0.84. This finding indicates that in the rat there are no significant shifts in the distribution of red cells and plasma to disturb the ratio. It indicates, for one thing, that repeating the experiment in the splenectomized rat would provide little additional information. Also, of course, with splenectomy one would run the risk of activating a hemolytic process due to Baronellosis.

Since neither plasma volume per 100 g nor TBH/LVH changes significantly, large-vessel hematocrit should correlate well with red cell mass per 100 g during the entire experimental period, over a substantial range of hematocrit. This correlation is seen in Fig. 7.

To put it another way, prediction of red cell mass from hematocrit and weight is excellent throughout the whole period of reacclimatization to sea level--but the weight gain cannot be ignored. The prediction can be done satisfactorily either with the preferred multiple linear regression of RCV on LVH and weight, or with regression of RCV/100 g on LVH and then simply multiplying by the weight.

The data from all three experiments in this section are best explained by passive destruction of red cells, that is, a normal erythrocyte life span on reacclimatization to sea level. If there is any active process it must be quantitatively rather small and of short duration.

Although Pace et al. measured only red counts in humans, and Reissmann measured red cell volumes with a plasma label, at the time of beginning of this work there appeared to be growing evidence for active destruction of erythrocytes.

Measuring red cell volume in mice, Alafi found a rate of decrease that could only be active.[8] However, the possibility of effects of Bartonellosis was great in his material, since he had splenectomized a number of them, and others were anemic without splenectomy, indicating clearly the precarious balance with Bartonella that was present in the mouse colony at that time. Also, Alafi's data are given in ml RBC per 100 g throughout. This appears to take adequate account of

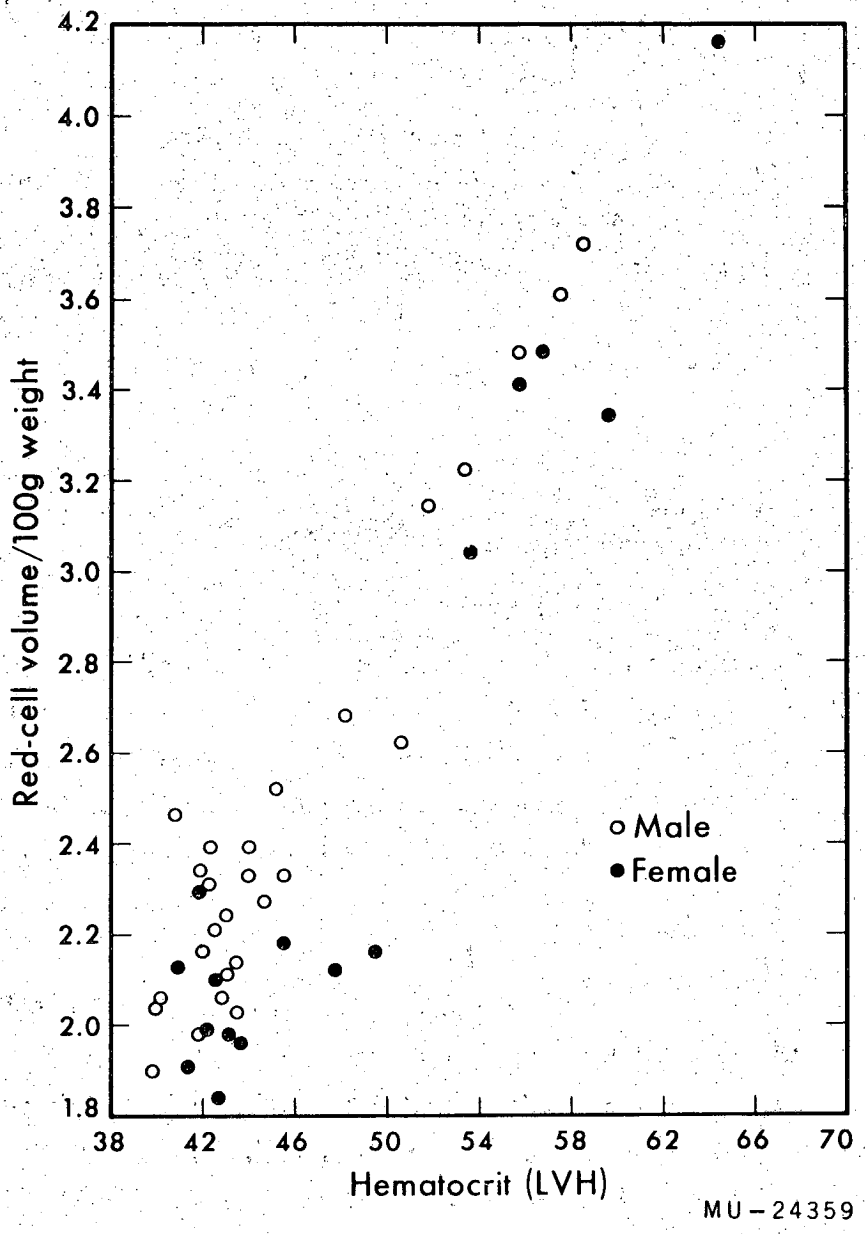

Fig. 7. Scattergram to indicate the correlation of red cell volume per 100 g body weight with hematocrit (LVH). Values from male and female animals in all experiments including altitude, fasting, and hyperoxia.

weight gain, but does not actually do so, as the above discussion indicates.

As mentioned above, Merino had also suggested on the basis of bilirubin excretion that there was active destruction in humans.[6] More recently Reynafarje has obtained normal life spans during descent,[1,2] using both C^{14} glycine and Cr^{51}, tending to confirm the original finding of Fryers and Berlin.[34] These latter two papers either did not measure red cell mass or did not record having done so. As mentioned above also, Van Dyke, in a monkey, with C^{14} glycine and frequent p^{32} RBC volumes, found no evidence for active destruction.[35]

In rats, with the methods described here, and with an accounting for total red cell mass per animal, we also find no evidence for any significant degree of active destruction.

As mentioned above, the test done here using DFP^{32} is somewhat different from that done by Fryers and Berlin in rats with C^{14} glycine. The DFP^{32} data indicate that cells of all ages in the peripheral blood on the day of descent are removed at a normal rate thereafter. The C^{14} glycine data, if corrected for changing red cell mass, would indicate the rate of removal of cells formed in the first few days after descent.

A Definition of Active Red Cell Destruction

The concept of active destruction of red cells can be defined most clearly and concisely (though not necessarily in conformity with all the intuitive ideas that may become attached to it) as follows.

Passive or purely age-dependent destruction conforms to the following integral equation, whereas active destruction requires a more complicated kernel, S, that is not solely a function of age:

$$M(t) = \int_{-\infty}^{t} R(\theta)\, S(t-\theta)\, d\theta$$

where

M (t) is total red cell mass at time t ,

$R(\theta)$ is red cell production rate at a previous time θ,

$S(t-\theta)$ is a function indicating surviving fraction (age $t - \theta$) at time t of those cells formed at time θ.

Although it actually says something quite different, the above equation closely resembles the one proposed by Shemin and Rittenberg for measurement of red cell life span with N^{15} glycine.[36,37] The same and similar equations for various metabolizing systems have been described by other authors.[38-42] Wijsman discussed the equation and pointed out the differences there must be in the equation of a purely age-dependent survival and of more complicated processes.[42] While the survival function S must of necessity be a function only of age $(t-\theta)$ when one uses it to describe only the normal steady state, as Shemin and Rittenberg used it, the assertion that this same function $S(t-\theta)$ (and therefore the same integral equation) remains unmodified in a particular circumstance (such as removal from the altitude chamber) constitutes the hypothesis that there is no active destruction. That is, the normal sea-level life span and life-span distribution still apply.

A consequence of the above definition that has been alluded to at times, but not explicitly pointed out, can be seen in Fig. 8. The figure is made up of a group of approximate solutions to the integral equation, using the data for red cell mass at altitude from Fryers.[43] It is intended as an illustration, not a precise prediction. The increase in red cell mass brought about by exposure to altitude is plotted. Then, starting at 0, 15, 30, 45, and 60 days, the descending curves indicate the theoretical rate of decrease of the population of cells present at each of those times. The curves are constructed assuming only an age-dependent survival with mean life 45 days and a normally distributed survival curve with 95% surviving to 35 days.

The descending curves represent either the decrease of the cell population uniformly labeled at one of the indicated times, or the

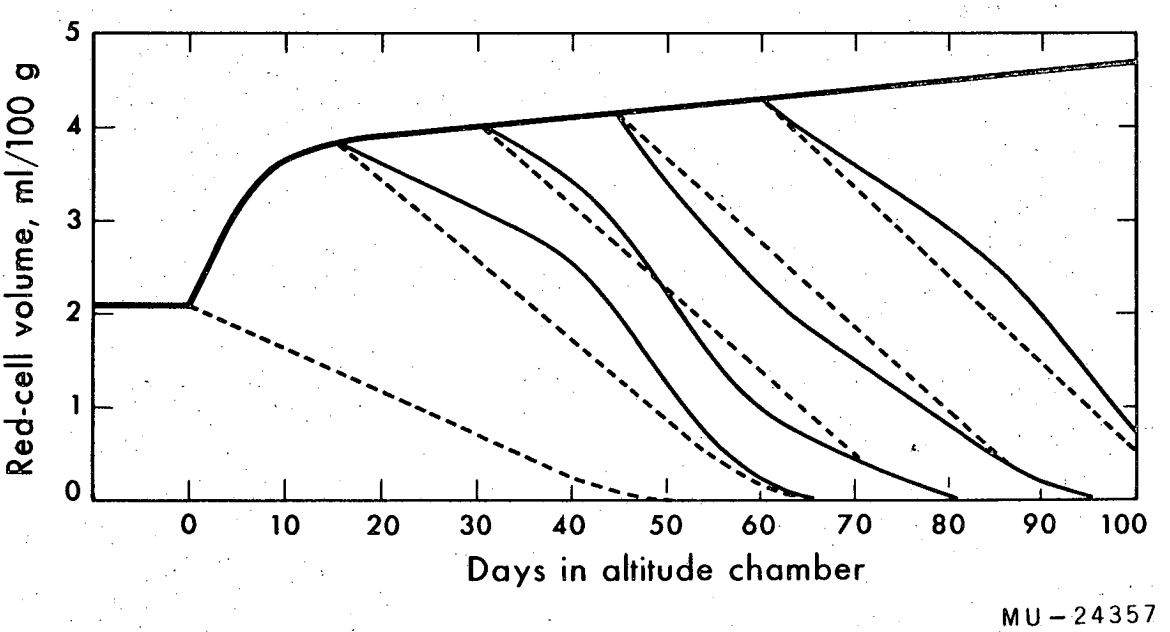

Fig. 8. Theoretical red cell volume curves. See text for explanation.

theoretical maximum rate of decrease of total red cell volume if production ceased entirely at that time.

The previous history of the animals's erythropoiesis affects the age distribution of red cells at the beginning of the descent, and as the figure shows, this markedly influences the shape of the theoretical curve of decrease of labeled cells, even in the absence of active destruction. The curve is deviated to one or another side of the linear decrease found in the steady state, depending on the time elapsed since the very rapid influx of new cells at the onset of exposure to altitude. The periodic variation in red cell production necessary to sustain this measured red cell volume in the face of crops of relatively uniform age cells reaching their full life span is shown in Fig. 9. There is a corresponding periodicity in the shape of the decreasing curves of Fig. 8. These periodic variation will eventually be damped out if the animal stays at altitude long enough to stabilize not only red cell volume but also red cell age distribution. The time this is likely to take is longer than one might have assumed, and longer indeed than that to which rats have been exposed in any published experiments.

A similar but reversed pattern of curves should theoretically follow descent from altitude or exposure to hyperoxia.

Also, if there were any nonuniformity of DFP labeling it would express itself in changes in these curves. A tendency to label young cells preferentially would further distort the curves of Fig. 8. A wider distribution of life span than the one arbitrarily selected would tend to lessen deviations from the linear decrease. Figure 8 therefore indicates some of the difficulties to be expected with details of hypothesis testing when attempting to test active versus passive destruction. So far the experiments done, including those reported here, have simply been planned to iron out such variations by keeping the animals at altitude fairly long, and to test against a theoretical linear decrease.

Thus difficulties of several kinds--in experimental design, gathering of data, and the mathematical work--have so far made detailed application of the integral equation a practical impossibility. But the equation does serve as an explicit definition of active destruction.

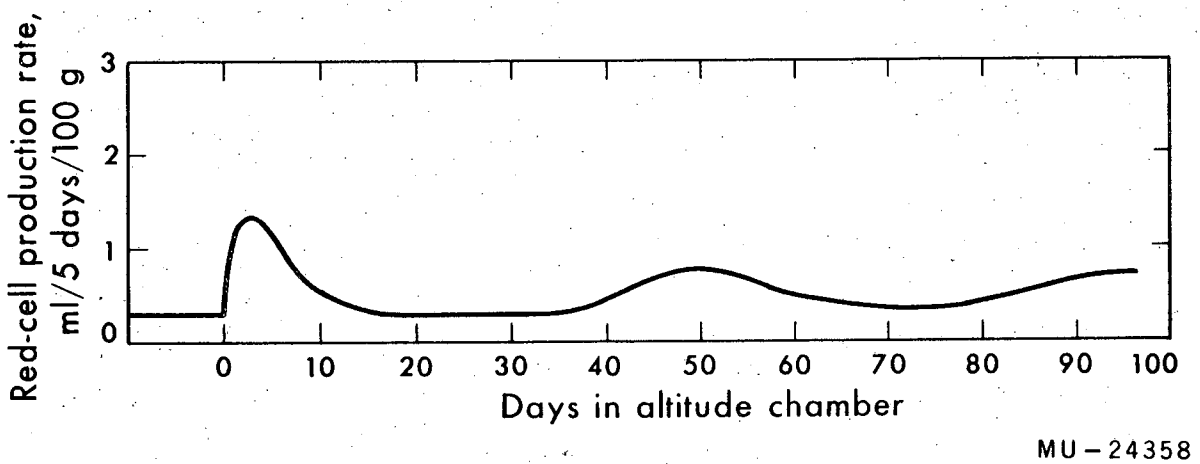

Fig. 9. Theoretical curve of red cell production rate. See text for explanation.

5. RED CELL VOLUME OF THE FASTED RAT

Introduction

This section outlines a study of the effect of a period of complete fasting on red cell volume, plasma volume, hematocrit, and the TBH/LVH ratio. Such data do not appear to be available elsewhere. It gives information on the effect that fasting might have on red cell mass, or the TBH/LVH ratio in the other sections of this report. As the data below indicate, these two parameters are sufficiently invariant with short periods of fasting that any such changes may be ignored in experiments requiring only a few hours' fast. Only some interaction of fasting with another effect remains possible.

Methods

Sixteen Long-Evans male rats weighing 220 to 320 grams were divided at random equally into control and experimental groups. The experimental group was fasted for 4 days, but allowed water ad lib. At the end of this period the combined red cell and plasma volume measurement was made by using the method of Section 2.

Results

Table III summarizes the findings. From the table it is evident that the fasted animals weighed 22% less than their controls. The decrease in plasma volume paralleled the over-all weight loss closely, while the red cell mass was not significantly decreased. The mean result was a 17% higher hematocrit. The mean ratio of total-body hematocrit to large-vessel hematocrit, 0.82, was unchanged by the 4 days of fasting.

Table III. Red cell values in adult LE male rats after 4 days of fasting.

	RCV	RCV per 100g	PV	PV per 100g	BV	BV per 100g	TBH	LVH	TBH/ LVH	Wt. at onset of fasting (g)	Wt. at day of measure-ment (g)	No. of rats
Control	5.82 ±.24	2.14 ±.10	10.6 ±.33	3.89 ±.08	16.4 ±.32	6.03 ±.09	35.5 ±1.4	43.4 ±0.6	0.82	262	272	8
Fasted	5.48 ±.22	2.62 ±.13	7.73 ±.25	3.68 ±.11	13.2 ±.40	6.30 ±.21	41.5 ±1.0	50.6 ±0.4	0.82	262	211	8
% Change	-5.8	+22	-27	-5.4	-19	+4.5	+16.9	+16.6	0	---	-22.4	---

6. ERYTHROKINETICS OF ACCLIMATIZATION TO HYPEROXIA

Introduction

My attention first turned to the erythrokinetics of acclimatization to oxygen partial pressures greater than normal because of the thought that changes in red cell mass observed after descent from high altitude might be accentuated by immediate removal from high altitude to as high an oxygen pressure as rats would tolerate. This, it was thought, would show active destruction in as striking a way as possible, if it occurred. There was also the possibility of characterizing the relationship of red cell volume to ambient oxygen pressure over a wider range than has been previously measured. Since the effect of prolonged exposure to hyperoxia on red cell mass of rats is considerably less than predicted from some previous animal exposures, the change from high altitude to hyperoxia was never carried out. However, the erythrokinetic pattern of the first month of exposure to 50% oxygen at 760 mm total pressure was explored.

The measurements fell into three independent experiments, (I) measurement of red cell mass with Fe^{59}-labeled rat red cells during the first month of exposure, (II) measurement of red cell formation with 24-hour Fe^{59} incorporation into red cells, and (III) measurement of red cell life span with DFP^{32}-labeled red cells.

In the postaltitude experiment, the pattern of Fe^{59} incorporation into red cells has been thoroughly established by other workers, but it was not available for hyperoxia for more than the first few days. Predicting the long-term pattern on the basis of available experiments was difficult. Therefore, even though Fe^{59} incorporation is only a semiquantitative technique in comparison with red cell mass and DFP^{32} turnover, it also was checked.

It has been clearly shown[44, 45] that continuous exposure to oxygen partial pressures greater than 456 mm (equivalent to 60% oxygen at 760 mm total pressure) gives rise to oxygen toxicity (see Discussion), a phenomenon with which it did not seem desirable to get involved. Higher oxygen tensions can be tolerated after a conditioning

period at 60% or by initially intermittent exposures.[46] However, in order to expose animals immediately to a concentration that they could then tolerate for weeks at a time, the work reported here was done with 50% oxygen at 760 mm total pressure.

Experiment I. Red Cell Mass

Bornstein in 1911 measured red cell count and hemoglobin on dogs and monkeys at total pressures of atmospheric air twice normal. She found a small decrease in both. In addition, in three dogs she measured total-body hemoglobin by a total-body perfusion technique, and came to the conclusion that the lowered red cell counts were due to hemodilution.[47] In 1927 Campbell found decreases up to 35% in red count and hemoglobin on exposure of rabbits, rats, mice, cavies and monkeys to oxygen pressures of 425 millimeters (60 per cent oxygen) at normal total pressures.[48, 49]

Boycott and Oakley in 1933 exposed rats to 65% oxygen for ten weeks.[32] They found normal hematocrits and hemoglobins, but a sustained decrease in reticulocytes which led them to imply the possibility that red cell life span might be increased, since red cells might be "used less" in hyperoxia.

In 1951 Cooperberg and Singer[50] measured hemoglobin, RBC, reticulocytes, and marrow erythroblasts after exposure of guinea pigs to 50% oxygen up to 36 days. They found evidence for a temporary decrease in production from reticulocytes and marrow findings, with a return to normal at the end of the period. They found a slight decrease in hemoglobin and erythrocyte values also, though not as great as Campbell had observed in rabbits.

Numerous other measurements of red count and hemoglobin have been made after short-term exposure to increased pO_2,[45] but apparently only the four quoted above were measured after weeks of exposure. Hence it seemed desirable to apply an isotope-dilution technique to the measurement of red cell volume in high pO_2 maintained for 1 to 4 weeks.

Detailed study of the rate of change of red cell volume during the first few days of exposure to high pO_2 turned out not to be feasible with any reasonable number of rats, as initial measurements showed, since the effect is small and the variability high. The study was therefore confined to measuring red cell volume between 7 and 28 days after exposure to high pO_2 began.

Methods

Male Long-Evans rats weighing 200 to 300 g were used. They were divided randomly into equal-sized control and experimental groups.

Experimental animals were housed in a container especially built for the purpose. In it they were kept continuously in an atmosphere of 50% oxygen–50% nitrogen for periods of 1 to 4 weeks. Some experimental animals were maintained in the same box, but supplied with ordinary compressed air. The box was made of laminated plywood, with a clear plastic face plate, and contained inlet and outlet fittings, a fan to maintain mixing of the atmosphere, room for two to four rat cages, and racks for drying materials. Although the box was relatively tight the outlet was kept open, and despite the high flow rate maintained, the interior of the box was continually at room pressure.

Inlet and outlet air could be sampled with a Beckman oxygen analyzer at any time, and were checked at least daily. Tanks of 50% oxygen–50% nitrogen continually supplied gas to the box at 3.5 liters per minute, and the measured outlet oxygen percentage was at all times more than 48% except during a 10- to 20-minute period daily for animal care. The volume of the box was 197 liters. Carbon dioxide concentrations were not measured. Calculations involving the metabolism of normal rats, the volume of the box, and the flow rate showed that the maximum value for carbon dioxide when ten rats were in the box should have been 1.2%. The experiment was done sometimes at this level and sometimes with five rats present, giving approximately 0.6% carbon dioxide. Because of the lack of direct measurement of CO_2, the somewhat high calculated value of CO_2, and the far-from-ideal removal of water vapor (by anhydrous $CaSO_4$ and silica gel and

by maintaining a fairly high flow rate), and also because of possible unknown factors, the additional control of exposing some animals to normal air at atmospheric pressure within the box was essential.

Since there was only one enclosure, oxygen exposures and compressed air exposures were done at different times, but each with control animals. At intervals, animals were removed from the box and, along with their control groups, sacrificed for the determination of red cell volume using Fe^{59}-labeled RBC from a donor rat. The technique previously outlined for the low-atmospheric-oxygen studies was used for red cell volumes and hematocrits. Blood volume was merely calculated from RCV and hematocrit rather than by the combined-labeling method used in other sections of this report.

Results

The principal findings are summarized in Table IV and Fig. 10.

Continuous exposure to 50% oxygen at a total pressure of 760 mm Hg for periods up to 28 days caused a small but statistically highly significant lowering of the red cell volume per 100 g. The red cell volume was 90% of the control value, and was not significantly different whether exposure had lasted only 7 days, or up to 28 days. Exposure to air at atmospheric pressure in the box resulted in no significant change in RCV per 100 g.

Statistical analysis was done by distribution-free methods using the Kruskal-Wallace one-way analysis of variance by ranks or the Mann-Whitney U test, as applicable.[51] It showed that the RCV/100 g in the oxygen-exposed animals was clearly different from either that of the control or air-exposed groups at the $p < .001$ level, while control and air-exposed animals' RCV/100 g were indistinguishable ($p \approx 0.5$). The hematocrit averaged 95% of the control. With the same statistical methods, hematocrit could only be established as different from control in any of the groups at the 90% level of confidence; weight gains during the experimental periods were not significantly different in any of the three groups ($0.30 < p < 0.50$).

Table IV. Red cell volume and hematocrit in LE male rats exposed to 50% oxygen.

Duration of exposure	RCV/100 g		Hematocrit		BV/100 g		Weight gain per 10 days		No. of rats	
	Expt.	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.	Control
				Oxygen						
7 days	2.11±.03	2.27±.07	43.0±.4	44.6±.8	4.91±.07	5.09±.14	24	24	5	3
10 days	2.06±.05	2.33±.04	42.8±.9	44.0±.7	4.78±.21	5.30±.13	16	37	4	5
14 days	1.90±.07	2.39±.05	39.8±2.5	44.0±.8	5.10±.22	5.39±.13	35	29	4	4
15 days	2.04±.05	2.21±.05	40.0±.8	42.5±.7	5.10±.15	5.20±.07	23	30	8	6
28 days	1.98±.12	2.03±.12	41.8±.9	43.5±1.1	4.75±.28	4.68±.16	27	27	4	3
				Air						
7 days	2.34±.13	2.31±.04	41.9±1.4	42.2±.7	5.58±.08	5.47±.05	31	31	4	5
13 days	2.46±.11	2.39±.08	40.8±.8	42.3±.4	6.03±.18	5.65±.15	28	28	5	5
				Combined mean values						
All controls	2.28±.03		43.2±.03		5.31±.06		28±2		31	
All oxygen	2.02±.03		40.9±.06		4.95±.09		25±2		25	
All air	2.41±.08		41.2±.9		5.83±.13		29±6		9	

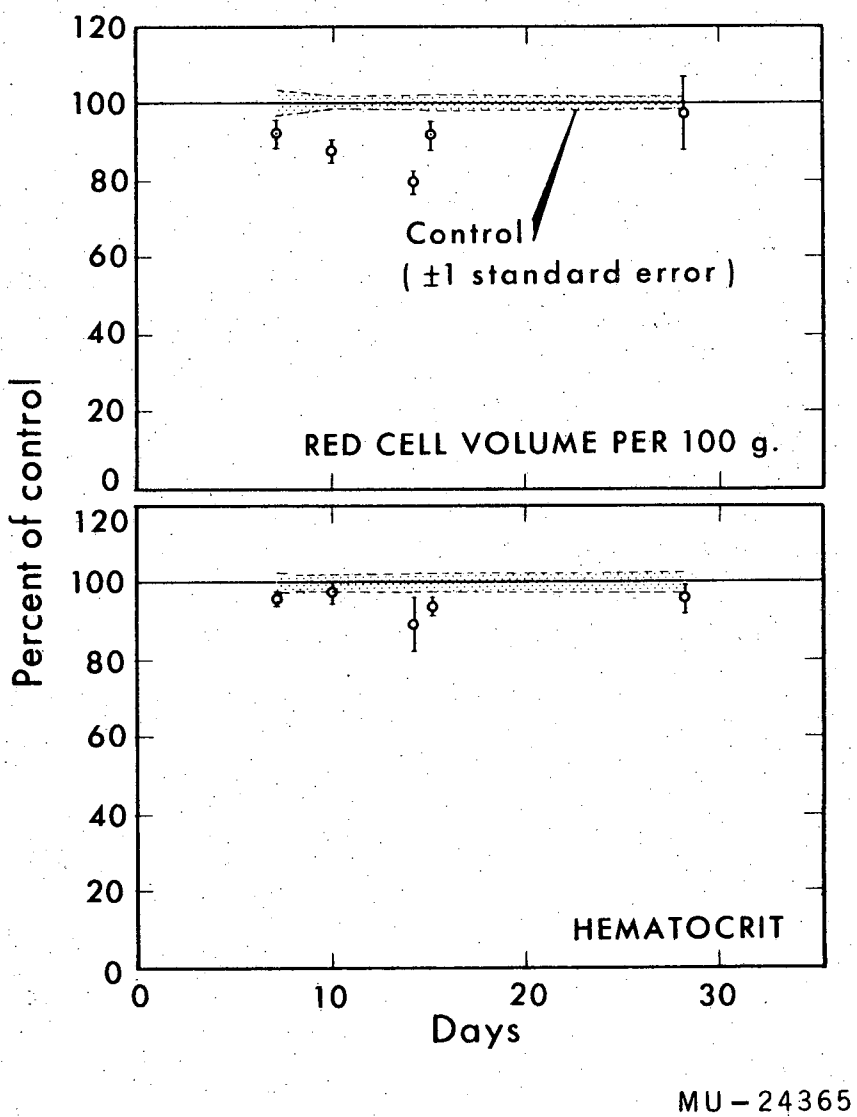

Fig. 10. Red cell volume per 100 g and hematocrit in per cent of control (± 1 standard error) for adult LE male rats exposed to 50% oxygen.

The blood volume, in this case calculated from red cell volume and hematocrit, was also of course decreased to 93% of the control value. Tested separately, this decrease was significant at the $p < .05$ level.

As mentioned above, the mean decrease in red cell volume was small between 7 and 28 days, and showed no trend of further decrease after 7 days -- in fact, possibly the reverse (see Fig. 10). The over-all decrease was established as statistically significant only with a sizeable number of animals.

Assuming a finite life span of 45 days and no red cell production whatever, one would predict the red cell volume to decrease at 2.2% per day, reaching 89% of control at 5 days, 78% at 10 days, and so on.

This experiment is inconsistent with any quantitatively significant amount of active destruction. It is consistent with a normal finite life span. It does not allow the detailed analysis of the kinetics of red cell production that would theoretically be given by frequent measurement of red cell mass during the first five days of oxygen exposure. However, it establishes that active destruction cannot be a very important mechanism if it occurs here, and that the great variability in material would prevent such detailed analysis from being very meaningful anyway. There is nothing in this experiment to support the notion that exposure to 50% oxygen leads to a rapid or random destruction of red cells in the normal animal.

The possibility of refining the calculation by combining data on red cell mass and Fe^{59} incorporation is considered below.

Experiment II. Iron-59 Red Cell Incorporation

Male Long-Evans rats weighing 200 to 300 g were again used. They were housed in the same container described in Experiment I, in an atmosphere of 50% oxygen–50% nitrogen for periods from 1 day to 1 month. Every effort was made to maintain conditions exactly as they were for Experiment I. Control animals were maintained outside the container as before. At intervals control and experimental animals

were injected intraperitoneally with a tracer dose of 0.4 μC of Fe^{59} previously mixed with rat plasma. They were immediately returned to the previous environment for 24 hours, and then sacrificed. Eight ml of aortic blood was obtained from each. Aliquots of whole blood and plasma were measured in the well counter and compared with standards for determination of per cent incorporation of the injected Fe^{59} into erythrocytes.

Results

The results are given in Table V and Fig. 11. Results are subject to considerable variation, as control values show in the table. The reduction of iron incorporation in per cent of the control was statistically significant between the third and the twelfth day. The average value during this period was 79% of control. At 17 and 31 days the average value was 125% of control.

Experiment III. DFP^{32} Red Cell Life Span

The rats and their maintenance in 50% oxygen, selection of controls, etc., were entirely comparable to Experiments I and II.

The methods employed in DFP^{32} injection and measurement were those described in Section 3 with ip injection and repeated small samples of tail-tip blood. The DFP^{32} was injected just before the rats were introduced into the box for their month's stay, thus labeling a population of cells of all ages formed prior to oxygen exposure, but permitting a study of their destruction rate during the exposure.

Total DFP^{32}-labeled red cell mass was calculated by multiplying the activity in counts/min/ml by the red cell mass found in Experiment I of this section. This total labeled red cell mass is displayed in Fig. 12. There is no detectable difference in the control and experimental curves.

Discussion

The three experiments of this section, taken together, indicate that exposure to hyperoxia for a month causes rat red cell mass per 100 g to decrease to 90% of normal control values. This is not simply

Table V. Fe^{59} incorporation in LE male rats exposed to 50% oxygen

Duration of exposure (days)	Fe^{59} Incorporation % of injected dose			Hematocrit			No. of rats	
	Expt.	Control	% of Control	Expt.	Control	% of Control	Expt.	Control
1	24.3±2.6	31.5±2.9	77.1±10.9	42.3±0.8	39.8±1.0	106.3±3.4	4	5
2	27.7±2.9	25.6±1.7	108.2±13.4	40.3±0.4	42.4±0.2	95.0±1.0	4	6
3	14.7±0.6	19.9±1.3	73.9± 5.7	41.8±0.4	43.4±0.7	96.3±1.8	6	6
4-1/3	14.7±1.5	23.7±1.6	62.0± 7.6	43.1±0.5	44.7±1.2	96.4±2.8	5	5
6	15.5±1.8	20.3±2.0	76.4±11.6	41.1±0.7	40.4±0.6	101.7±2.3	3	6
8	18.9±1.1	22.8±1.1	82.9± 6.3	40.7±1.1	45.0±0.7	90.4±2.8	5	6
12	23.8±1.4	32.7±2.3	72.8± 6.7	39.7±0.4	41.6±0.3	95.4±1.2	6	5
17	29.8±1.3	22.3±3.0	133.6±18.9	39.1±0.7	44.2±1.2	88.5±2.9	5	5
31	28.5±2.0	24.5±2.7	116.3±15.2	38.3±0.9	42.1±1.2	91.0±3.4	6	4

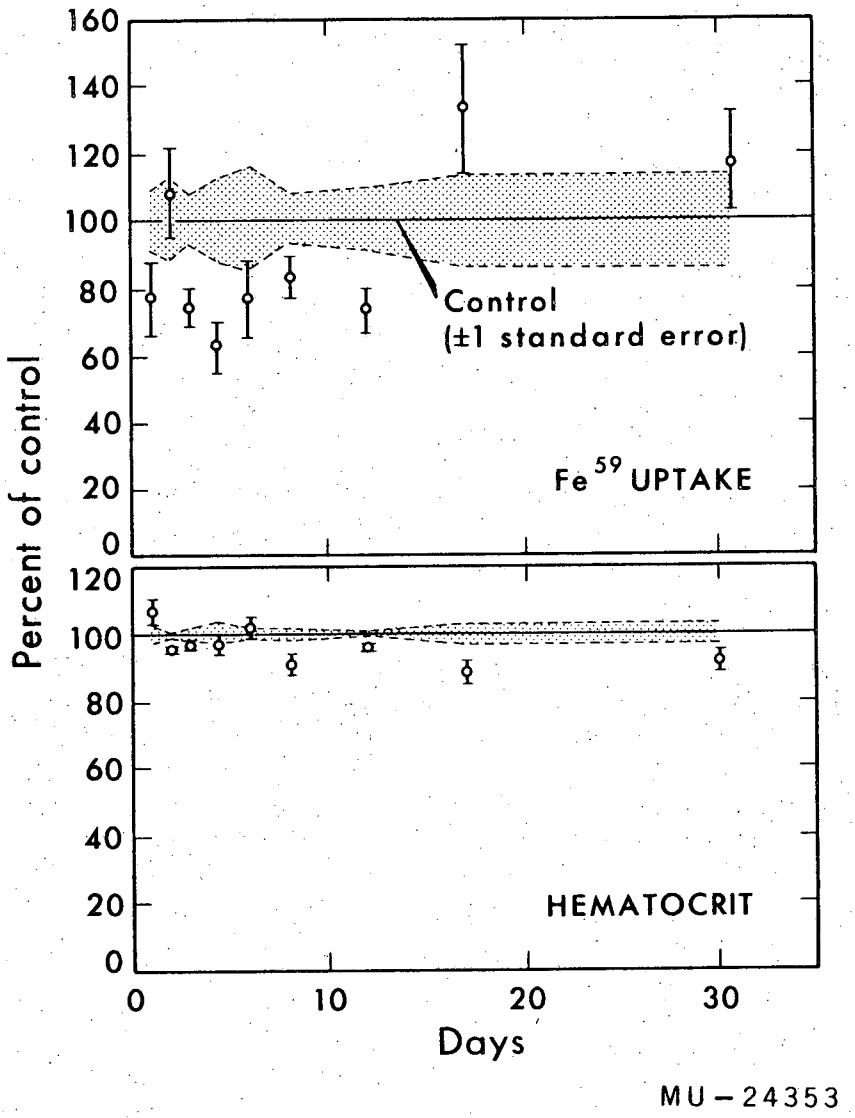

Fig. 11. Incorporation of 24-hour Fe^{59} into red cells, and hematocrit (per cent of control ±1 standard error) in adult LE male rats exposed to 50% oxygen.

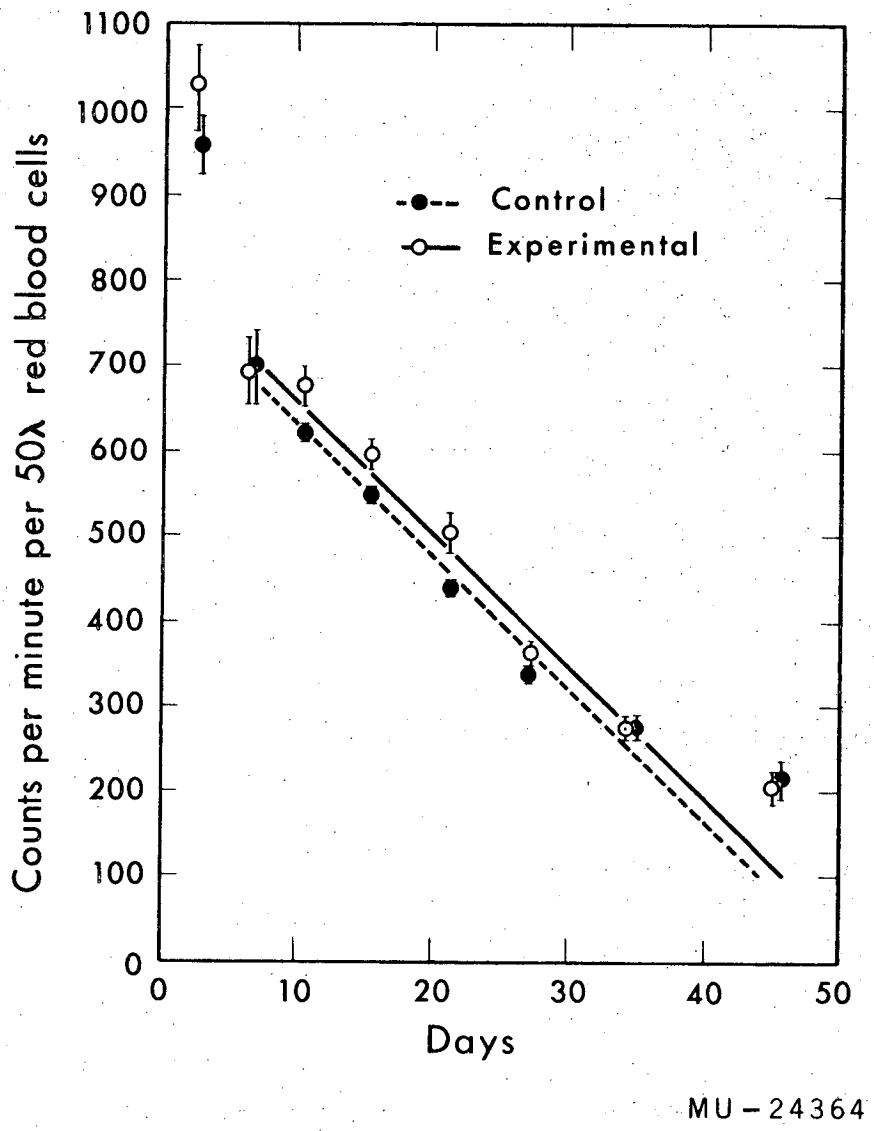

Fig. 12. DFP^{32} red cell life span of adult male LE rats exposed to 50% oxygen and their controls (±1 standard error). Least-squares lines fitted to points from 4 days through 32 days.

a reflection of weight changes. The red cell mass is reduced 10% by 1 week and subsequently stays at 90% of normal. Red cell iron incorporation is reduced to approximately 80% of control between the third and twelfth days, and then returns to control levels or greater. Red cells of all ages present in the circulation at the beginning of the exposure are removed at a rate indistinguishable from control. The temporary decrease in red cell production probably entirely explains the decrease in red cell mass during the first week without need to postulate an increased destruction rate.

Actually, if production is three-fourths normal, the red cell mass should fall at only 1/4% per day, taking at least 40 days to reach 90%. But the iron incorporation procedure is unfortunately not a sufficiently quantitative estimate of rate of delivery of mature cells to the peripheral blood to rely heavily on it to quantitatively account for the small change in red cell mass (see Discussion, Section 4).

In retrospect these three experiments tell very little about the nature of this small readjustment in red cell mass per 100 g body weight that takes place in the first week. They do indicate that there is such a change, that it is small, and that it persists for at least a month. By the end of the first month iron incorporation is no longer depressed, and red cell turnover is comparable to the control value.

The relationship of red cell mass and hematocrit to ambient oxygen pressure at the three pressures used in this paper are shown for Long-Evans male rats in Fig. 13.

Since the proportionality of red cell mass to oxygen tension is quite well established by numerous workers for points between sea level and 15,000 feet, [1, 52] the sharp bend in the curve at sea level is probably real. The bend is somewhat sharper than with Campbell's data on hematocrit and red counts on rabbits. [49]

Our data agree well with those of Boycott and Oakley in rats, [32] Cooperberg and Singer in guinea pigs. [50] It is easier to reconcile than is Campbell's finding of a 60% red cell count with the notion that red cell mass is regulated somehow so as to make it easy to maintain

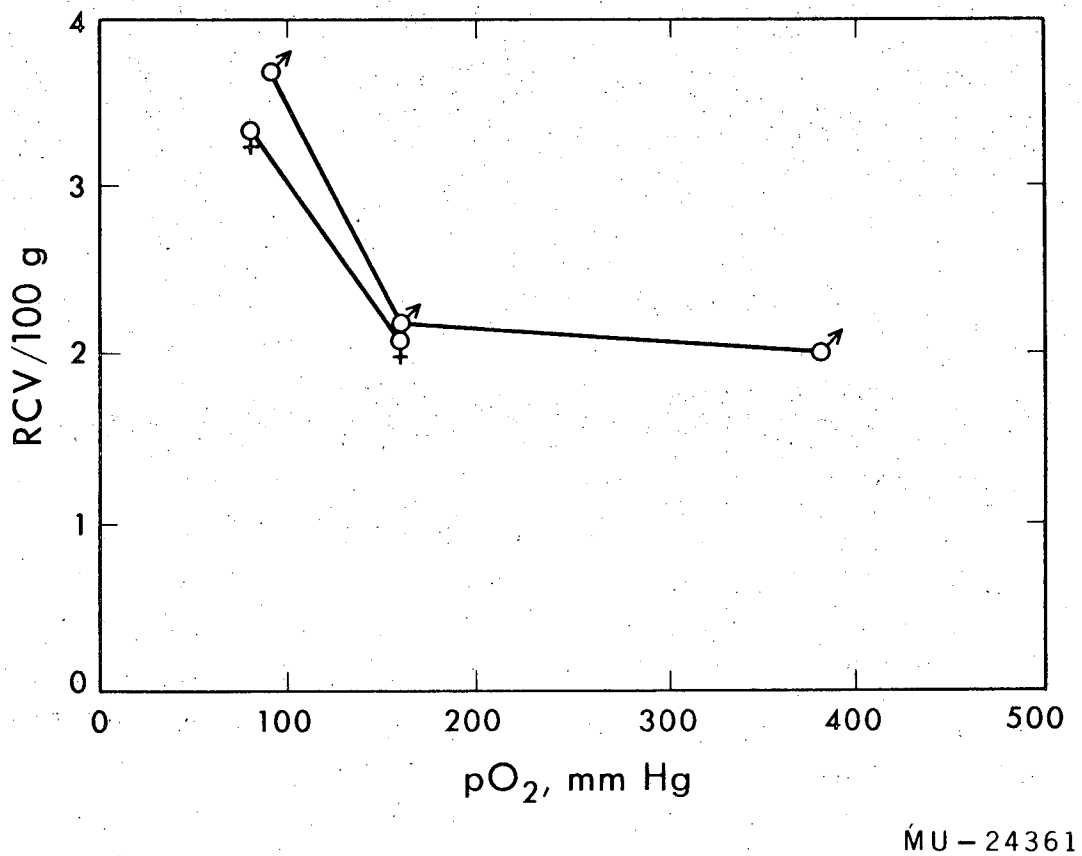

Fig. 13. Red cell volume per 100 g body weight plotted against ambient oxygen partial pressure for adult LE rats at simulated high altitude, at sea level, and at high oxygen pressure. Mean values for "fully acclimatized" animals in this series of experiments.

oxygen delivery, while returning resting cardiac output eventually to preacclimatization values. No studies of cardiac output are available on prolonged hyperoxia. Campbell has shown that oxygen consumption remains normal.[48,49] Whether oxygen delivery and extraction remain normal is not known, although significant changes in them seem unlikely.

It appears, extrapolating from the available data in humans,[53,54] that the 5% decrease in arterial hematocrit found in rats maintained on 50% oxygen is just sufficient to compensate the increase in dissolved oxygen and hemoglobin saturation, making possible a normal oxygen delivery with no change in cardiac output.

The changes in hematocrit induced by full acclimatization to high altitude are also approximately such as to preserve normal delivery with normal cardiac output. To quote Boycott and Oakley[33] on this subject,[32] "Experience shows . . . that persistent changes in the oxygen supply, however produced, are always in the end associated with alterations in the concentration of hemoglobin in the blood unless some other consideration (e.g. heart failure) overides the normal response. One surmises therefore that this is the method of choice and that the animal prefers, when any first emergency is over, to revert to its customary method of gaseous exchange in the lungs, its usual circulation rate and its normal blood reaction." The consistency of this pattern is interesting and may seem meaningful teleologically, but at present does not particularly assist in understanding either the body's adaption to a new tissue oxygen tension, nor the mechanism of the 10% decrease in red cell volume that appears as a part of the adaptation.

There is some confusion in the literature on the effects of prolonged oxygen administration which these experiments can help to clarify. As mentioned above, it is now well established that chronic exposure to oxygen partial pressures greater than 456 mm Hg (equivalent to 60% oxygen at 760 mm total pressure) gives rise to "oxygen toxicity," with evidence of pneumonitis after 3 to 4 days or of central nervous system damage sooner, depending on the concentration. Oxygen concentrations somewhat higher than 60% can be tolerated after a

conditioning period at 60% or by initially intermittent exposures. In human studies, prolonged exposure to 60% or less has proven to be safe.[44]

However, there has been some implication in recent work that erythropoiesis is decreased on exposure of normal humans to hyperoxia, and the subsequent events--whether there is a permanent decrease in red cell mass, or a return of erythropoiesis to normal, for example--have not been clearly worked out.

Reinhard et al.[55] and Tinsley et al.[56] showed the following evidence for depressed erythropoiesis during fairly prolonged hyperoxia: three people with chronic hemolytic anemia had a dramatic decrease in reticulocytes, a fall in red cell counts, and a decreased Fe^{59} red cell incorporation on a 2-week period of 50% oxygen by mask. After oxygen was discontinued, a marked reticulocytosis occurred, red cells returned rapidly to control level, and Fe^{59} incorporation increased. In four untreated cases of pernicious anemia, B_{12} injection caused small reticulocyte responses under the 2 weeks of 50% oxygen. In each case a larger second reticulocyte response occurred to B_{12} given after oxygen was stopped.

Two normal subjects in their study received 70% oxygen by mask for 10 days. They each had a decreased Fe^{59} incorporation into red cells, and a small but definite decrease in reticulocyte percentage. Several weeks after oxygen was stopped, each of them had an Fe^{59} incorporation within the normal range. Tinsley et al. state that their study indicates that high tensions of inspired oxygen depress erythropoiesis in humans, and provides further evidence that arterial oxygen tension is one of the principal regulators of erythropoietic balance. However, their study has uncertain implications for erythropoietic balance in a truly chronic exposure to 50% oxygen, particularly in normals.

Lawrence et al. found that 50% oxygen administered for 5 to 7 days to two patients with secondary polycythemia caused a decrease in plasma iron disappearance rate, while in four patients with polycythemia vera there was no decrease.[57] They reviewed the work to

that date (including that of Tinsley et al.) that indicated that the rate of red cell production is decreased in normal animals and humans by increased ambient oxygen. However, they made no measurements in normals.

Our study described above, and those of Boycott and Oakley[32] and Cooperberg and Singer[50] are concerned with a more prolonged exposure to hyperoxia, and the over-all findings are contracry to the implications of the 5- to 10-day studies by Tinsley et al. and Lawrence et al. They are also contrary to the long-term studies of Campbell in rabbits showing a decreased red count, but which did not include measurement of red cell mass. In addition, the hypothesis that red cell life span might be prolonged, drawn by Boycott and Oakley from their data on reticulocytes, has not been sustained by our DFP red cell life-span measurements.

The over-all implication of the studies on erythropoiesis in hyperoxia reviewed above would seem to be as follows: in normal animals the decrease in erythropoiesis is temporary, and the decrease in red cell mass is slight. Truly prolonged exposure to 50% to 70% oxygen is unlikely to be dangerous, or to require much (for normals) in the way of reacclimatization on return to sea-level oxygen tension. (At least this seems so from the point of view of cardiovascular and erythropoietic adaptation.)

The finding that with hemolytic anemias oxygen administration causes a decrease in red cells and a suppression of erythropoietic activity, while it does not suppress marrow response to hemorrhage,[50] indicates some fundamental property of the homeostatic system that has not yet been adequately investigated.

Data on prolonged exposure to increased ambient oxygen may some day have importance in space travel or therapy of chronic diseases. Also, such data must eventually be explained by any theory purporting to describe how red cell mass is matched to body metabolic needs--in hypoxia, hyperoxia, hypophysectomy, cold exposure, etc.

ACKNOWLEDGMENTS

The author gratefully acknowledges the technical assistance of Mrs. Lenore Bravo, Mrs. Elsa Isaac, and Mrs. Mary Chupp, and assistance with preparation of the manuscript by Miss Mary C. Wales.

The guidance given by my thesis committee, Dr. Ernest L. Dobson, Professor S. F. Cook, and Professor Hardin B. Jones, is very much appreciated.

This work was done under the auspices of the U. S. Atomic Energy Commission, and was partially supported by a Postdoctoral Fellowship of the National Heart Institute, U. S. Department of Health, Education, and Welfare.

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