EFFECT OF ANOXIC ANOXIA ON MYOGLOBIN CONCENTRATION IN STRIATED MUSCLE

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The characteristics of myoglobin in vitro have been demonstrated to be those of a respiratory pigment, but the rôle of myoglobin in vivo has yet to be fully established. The literature to date affords two substantiated concepts with regard to its physiologic nature; namely, that myoglobin exists in striated muscle as a distinct heme pigment, and that it may act as a short-time oxygen store in the cell to tide the muscle over from one contraction to the next (1).

The study here reported was undertaken to determine whether or not myoglobin plays a part in the adaptation response of the organism to chronic hypoxia. Specifically, the study was designed to elucidate the physiologic effect of high altitude hypoxia on the myoglobin concentration in specific muscles of albino rats, and to disclose the extent, if any, of parallelism between changes in the myoglobin content of muscles and the increase in hemoglobin content of blood which occurs because of altitude hypoxia.

EXPERIMENTAL

Albino rats were subjected to a simulated altitude of 25,000 feet for four consecutive hours daily, from 12 to 312 days. The condition of the animals and their adaptation response to exposure were ascertained by periodic physical inspection, and by body weight, blood hemoglobin content and hematocrit determinations. The hematocrit values were obtained with Van Allen tubes. Hemoglobin concentrations were determined by hemolyzing one part of whole blood from the tail in 250 parts of distilled water, centrifuging, and measuring the densities of the resultant solution of oxyhemoglobin with a Beckman Spectrophotometer at the wave lengths 5100, 5410, 5600 and 5770 A. The value for hemoglobin recorded was the average concentration calculated from the four densities for the wave lengths above, using the specific extinction coefficients for oxyhemoglobin at those wave lengths determined by Horecker (2).

Except for the daily periods spent at altitude, the exposed animals and their designated controls were caged together and kept on the same diet, to which they had unrestricted access. After specific periods of exposure to simulated altitude, pairs of experimental animals and their controls were killed. The gastrocnemius and soleus muscles were dissected out and analyzed for their myoglobin and hemoglobin contents by methods of analysis (3, 4) reviewed here insofar as they pertain to the data presented.

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1 A Beckman Spectrophotometer was used throughout this study. All measurements were made with a slit width of .04 mm.
The tissue sample was frozen in dry ice and hammered to a fine powder. A weighed amount of the powder was homogenized with a known volume of distilled water in the ratio 1.2 to 2.4 gm. of sample per 10 ml. H₂O. The homogenate was centrifuged to remove the extracted meat residue, and the supernatant decanted and heated rapidly in a water bath to a temperature of 53-55°C. Within the limits of pH and temperature stated, proteins producing turbidity in the supernatant were coagulated and removed by centrifugation and filtration without significantly affecting the concentration of myoglobin in the extract.

The cleared extract was buffered with M/2 phosphate buffer pH 7.1-7.2 and divided into two portions.

a) The chromoproteins in one portion were oxidized by the addition of a few particles of powdered potassium ferricyanide, and converted to cyan-metmyoglobin and cyan-methemoglobin by the addition of a small crystal of KCN. The sum total of myoglobin and hemoglobin in the mixture was calculated from the density of this preparation at the wave-length 540 μm by means of the formula, 

$$c = \frac{D}{E \times L} \times \text{d.f.},$$

where c is the total concentration of chromoproteins in moles or equivalents per liter, D is the optical density of the solution at 540 μm, E is Drabkin's molar extinction coefficient for cyan-metmyoglobin at 540 μm, L is the thickness of the absorbing layer, and d.f. is the dilution factor due to buffering.

b) The chromoproteins in the second portion were reduced with dithionite in an atmosphere of carbon monoxide to convert the chromoproteins to a carbonyl-myoglobin and carbonyl-hemoglobin mixture and the absorption of the preparation in the visible range was studied for indications of chromoprotein denaturation and, in their absence, for the specific wave-length densities required to calculate the heme pigment fractions in solution (fig. 1).

The molarities for the individual hemoglobin and myoglobin components of the extract were calculated from the densities at the wave lengths 568 μm and 538 μm, by means of the formulas,

$$[\text{MbCO}] = \frac{D_{568} \times E_{568}^{\text{MbCO}} - D_{538} \times E_{538}^{\text{MbCO}}}{E_{568}^{\text{Mb}} \times E_{538}^{\text{Mb}} - E_{568}^{\text{Mb}} \times E_{538}^{\text{Mb}}} \times \text{d.f.},$$

and

$$[\text{HbCO}] = \frac{D_{568} \times E_{568}^{\text{HbCO}} - D_{538} \times E_{538}^{\text{HbCO}}}{E_{568}^{\text{Hb}} \times E_{538}^{\text{Hb}} - E_{568}^{\text{Hb}} \times E_{538}^{\text{Hb}}} \times \text{d.f.},$$

where [MbCO] and [HbCO] are the molar concentrations of carbonyl-myoglobin and carbonyl-hemoglobin; D₅₆₈ and D₅₃₈ are the measured densities of the solution containing MbCO and HbCO at wave lengths 568 and 538 μm, respectively; E₅₆₈ and E₅₃₈ the molar extinction coefficients of 14.5 X 10⁻³ and 14.8 X 10⁻³ for MbCO at the wave lengths 568 and 538 μm, respectively; E₅₆₈ and E₅₃₈ similarly the molar extinction coefficients of 11.8 X 10⁻³ and 14.8 X 10⁻³ for MbCO at the wave-lengths 568 and 538 μm, respectively (fig. 1); d.f., the dilution factor for correcting dilution due to buffering, and the thickness of the absorption layer is 1.00 cm. (6).

In order to compare the myoglobin or hemoglobin determinations on different samples, the calculated molar concentrations were converted to milligrams of Mb or milligrams of Hb per gram of muscle by means of the formula,

$$\frac{\text{Mb mg/gm. muscle}}{17,000 \times [\text{MbCO}] \times \text{w.f.}}$$

and

$$\frac{\text{Hb mg/gm. muscle}}{17,000 \times [\text{HbCO}] \times \text{w.f.}},$$

where 17,000 is the equivalent weight assumed for Mb and Hb, and w.f. is the fraction of a liter equal to the sum of water used to transfer the powdered tissue sample to the homogenizing tube plus 75 per cent of the sample weight, assuming that 75 per cent is the water content of the muscle sample.

Identical processing was employed for each pair of experimental and control animals in the series to make their results directly comparable for the effect of altitude.

The quantity of cytochrome in rat gastrocnemius and soleus muscle extracted by this process is negligible.
exposure. To make the findings for all pairs directly comparable, the concentrations of myoglobin for each exposed animal and for its specific control are expressed in the form of a whole number ratio (table 2, col. 6). By thus equating all variables but the duration of altitude exposure for the different pairs, each ratio expresses a decreased or increased myoglobin concentration for the exposure time given in column 4, depending upon whether the ratio is less or more than 1.00. Comparison of the ratios for two or more pairs of animals gives an index to the relative changes in myoglobin concentration due to the differences in time spent at altitude.

With suitable modifications of the method reviewed above, analyses were made also of the hearts of animals numbered 6 to 12 in this study. Refinement of the modified method for rat heart analysis was carried to a point where significant but only roughly quantitative results were obtained. These preliminary data appear to

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Fig. 1. Absorption curves for carbonyl-myoglobin (MbCO) and carbonyl-hemoglobin (HbCO) for the wave-length span 536-578 μ. Determinations of the carbonyl-myoglobin and carbonyl-hemoglobin concentrations for mixtures of both heme pigments were based on the optical densities of the mixture and the respective molar extinction coefficients of the pigments at the wave length 538 and 568.

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3 Modifications in the method described were required to make it applicable for the analysis of heart muscle. a) Heat coagulation of the interfering proteins in a water extract of cardiac muscle does not take place within the temperature range given above for skeletal muscle extracts. This is due in part to the greater alkalinity of the heart extract (pH 6.75–6.90) as compared with that for the
corroborate the observation reported for heart tissue by Hurtado et al. (8) that the concentration of myoglobin in cardiac muscle is increased by high altitude hypoxia. Despite the significance of a study of myoglobin in heart muscle, this was not the primary purpose of the investigation. As a consequence, and because of apparent corroboration by the preliminary findings of observations already published, further attempts at methodologic refinement for more precise quantitative estimations of cardiac myoglobin were postponed. The observations made pertaining to cardiac myoglobin concentration and the implications drawn from these observations are mentioned in the following sections, but no pertinent quantitative data are given in this report.

**RESULTS**

**Body Weight, Hemoglobin and Red Cell Volume Changes**

These changes in the albino rat following altitude exposure have been dealt with intensively by other investigators (9-12). The uniformity of these changes in the exposed animals was such that the data for a single pair, given in table 1, may be regarded as typical. Increases in the red cell hematocrit values and hemoglobin concentrations began soon after exposure to altitude was initiated, and continued for approximately 6 to 10 weeks during the course of exposure before showing indications of having reached a steady state. There were no exceptions to the trend of these changes in any of the exposed animals.
Hemoglobin Content of Muscle Samples

The concentration of blood hemoglobin in the tissue extracts of animals exposed to altitude increased progressively with the duration of altitude exposure and paral-

### Table 2. Effect of Altitude Hypoxia on Myoglobin Concentration in the Gastrocnemius and Soleus Muscles of Albino Rats

<table>
<thead>
<tr>
<th>RAT</th>
<th>SEX</th>
<th>TERMINAL AGE, DAYS</th>
<th>DAYS OF EXPOSURE TO 25,000 FT. ALT., 4 HRS/DAY</th>
<th>CONC. OF MYOGLOBIN (Gastr. and Soleus)</th>
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<tr>
<td></td>
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<td>Mg/gm. of sample</td>
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<tr>
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<td>47</td>
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<td>i3X</td>
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**Miscellaneous Analyses**

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<tr>
<td>D</td>
<td>F</td>
<td>330</td>
<td>0</td>
<td>1.58</td>
</tr>
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</table>

1 'X' and 'Exp.'—Exposed animal. 'C' and Contr.—Control animal. 2 M. Gastr. only.
3 Early pregnancy.

led the rise in red cell volume closely. The hemoglobin concentrations for the animals 1X, 2X, 3X and 4X, exposed from 14 to 47 days, were 0.25, 0.41, 0.65, and 0.44 mg/gm. of muscle sample respectively. The hemoglobin values for the muscle samples of animals that reached a steady state, i.e. the group in which the exposure
period was 152 days or longer, ranged from 0.57 to 0.68 mg/gm. of muscle. The range of values for their controls was 0.08 to 0.20 mg/gm.

Although absolute values are used to express the concentration of "tissue-trapped hemoglobin", these values should be considered only as roughly quantitative in significance. They do demonstrate that, all other conditions being the same, the animal with polycythemia had an increased amount of red cells trapped in its muscle sample at death as compared with the control; the amount of hemoglobin present having a positive correlation with the duration of altitude exposure and red cell volume increase.

Changes in Myoglobin Concentration

1. Gastrocnemius and soleus muscles (table 2, cols. 5 and 6). The analyses of the animals with 152 days or more of altitude exposure all show marked diminution of myoglobin concentration in the gastrocnemius and soleus muscles, regardless of the sex of the animal or the age at which exposure was initiated. No significant change in myoglobin concentration could be demonstrated for the group with an exposure period of from 12 to 47 days however.

   It is interesting to note that of all the analyses made, the lowest myoglobin concentration in leg muscle was obtained on analysis of rat 13X (table 2). This one animal died during an exposure period at 25,000 ft. in the altitude chamber, after 198 days of previous exposure.

2. Cardiac muscle. The hearts of all exposed animals were grossly hypertrophied as evidenced by their size and weight. This condition due to altitude hypoxia has been previously described (9-12).

Heart analyses for changes in myoglobin concentration were made only for the animals numbered 6 to 12, of the group with 152 or more days of altitude exposure. In every instance the concentration of myoglobin per gram of heart muscle was significantly higher in the exposed animal than it was in the control. The findings therefore indicate both an increased concentration of myoglobin per gram of heart muscle, as well as an increase in the total content of cardiac myoglobin for the exposed animal.

DISCUSSION

The contrasting points of significance enumerated above may be summarized as follows:

1. There is no demonstrable change in concentration of myoglobin in the gastrocnemius and soleus muscles of rats exposed to altitude anoxia for periods up to 47 days, under the experimental conditions described. However, marked increases in blood hemoglobin concentration and circulating red-cell volume do occur during those periods.

2. There is a marked decrease in myoglobin concentration in the gastrocnemius and soleus muscles of rats exposed for 152 days or longer. However, the blood hemoglobin concentration and red-cell volume, after continuously increasing during the first 6 to 10 weeks of exposure, remain fairly stable thereafter and show little sig-

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5 The age at which altitude exposure was initiated is the difference between the total days of exposure for the animal and its terminal age; ref. cols. 3 and 4, table 2.
significant variation during the period of exposure in which a reduction in concentration of leg muscle myoglobin is observed.

3. Heart tissue analyses indicate an increase both in content and concentration of cardiac myoglobin in the rats exposed to altitude anoxia. Analyses of skeletal muscle, on the other hand, show a markedly decreased myoglobin content following prolonged exposure.

Exposure to anoxia was made in a manner that called forth no increased activity from the gastrocnemius and soleus muscles of the rats at altitude. In fact, the possibility is present that activity of those muscles may have been somewhat inhibited by chronic intermittent anoxemia. It has even been reported that skeletal muscles under anoxic conditions have a greatly lowered capacity for energy production; in other words, they are capable of much less work at altitude than at sea level (13a).

Cardiac activity, on the other hand, is greatly augmented by exposure to anoxia; at first by a physiologic demand for an increased heart rate and cardiac output and, subsequently, by the added burden of an increased blood viscosity which is only partially compensated for by changes in the vascular system. The high red cell volume and hemoglobin content of the typical example in table 1 illustrate this hemodynamic burden imposed on the heart by prolonged altitude exposure. The cardiac hypertrophy observed in all exposed animals demonstrates a phase of the adaptation response to the increased activity imposed by these factors on the heart. Since myoglobin content of striated muscle increases with muscular activity (14a, 15), contrasting variations of myoglobin content in skeletal and cardiac muscle following altitude exposure, as well as the findings noted in 1, 2, and 3 above, suggest: a) that changes of myoglobin concentration in a specific muscle arising after exposure of an animal to anoxia depend on the change in activity of that muscle due to the effect of the anoxic environment on the animal; and b) that myoglobin concentration is not altered by the mechanism causing hemoglobin variations, nor do observed changes in myoglobin concentration appear to be direct anoxic adaptation responses.

An investigation by Whipple (14b) demonstrated that there is no direct parallelism between the hemoglobin and myoglobin concentration in dogs following severe anemias experimentally induced and prolonged by repeated bleedings. The present investigation indicates that the lack of parallelism between changes in concentrations of hemoglobin and myoglobin extends also into the range of polycythemia due to altitude exposure.

Hurtado and his associates, on the other hand, report that parallelism does exist between the myoglobin level and the hemoglobin level in polycythemia of altitude observed in dogs. They suggest that a myoglobin increase in such cases represents an important mechanism of adaptation to chronic anoxemia at the tissue level (8).

Their observations, made on dogs native to altitudes of 12,300 and 14,890 feet, cannot be compared directly with those herein reported, because of the obvious differences in the method of exposure to altitude, the altitudes at which exposure was made, the difference in species of animal used etc. Dill has occasion to mention the adaptability and phenomenal capacity for activity of dogs at high altitude (13b). The differences in our findings thus may be due in part to the different capacities
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for activity at altitude of the animals employed. Nevertheless, the interpretation of Hurtado et al. that an increased concentration of myoglobin in dogs at altitude may represent a mechanism of adaptation to a condition of chronic anoxia, is one that cannot be reconciled with the findings here reported.

SUMMARY

A study was made to determine whether or not myoglobin plays a part in the adaptation response of an organism to chronic anoxemia. Quantitative estimations of the myoglobin in specific muscles were made on a series of albino rats exposed regularly but intermittently to a simulated altitude of 25,000 feet, for 12 to 312 days. The method of analysis employed is one which permits quantitative determinations of myoglobin to be made on extracts of unperfused muscle containing hemoglobin.

The data obtained indicates no direct relation between changes in myoglobin concentration and adaptation to a condition of altitude hypoxia. Skeletal muscle, in which activity is not enhanced by anoxia, eventually showed a decreased myoglobin content following prolonged altitude exposure; whereas cardiac muscle, in which activity is enhanced by anoxia, apparently showed an increased myoglobin content. These changes tend to indicate that the myoglobin content of a muscle is determined by muscular activity rather than by anoxia, even in an anoxic environment.

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REFERENCES

11. ALTLAND, P. D.  To be published.