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THE UNIVERSITY OF ALBERTA

INDICES OF HYPOXIA-INDUCED FREE RADICAL ACTIVITY IN TISSUES OF RATS

by

RAYMOND EINAR LOVLIN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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.....W.H. Cottle.....
(Supervisor)

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Date September 7, 1988

Dedication

I would like to dedicate this thesis to the memory of my parents, John and Margit Lovlin, my brother Jarle and my nephew Brad.

Abstract

The possibility that hypoxic conditions within tissues may enhance injury by oxygen radicals (free radicals) has been indicated by several observations. Levels of several substances in tissues, including increases in malon-(di)aldehyde (MDA) and lipofuscin and a decrease in cytochrome P-450, have been recognized to reflect peroxidation of lipids or proteins by the action of free radicals. Levels of these substances in tissues have been used by several authors as indices of tissue damage by free radicals.

These indices were determined in several tissues of rats following exposure to different types of hypoxic atmospheres (hypobaric versus normobaric-low in oxygen). The effect of duration of the exposure to a hypobaric atmosphere on these indices were also compared.

Other indices including edema, hematocrit, and methemoglobin which are known to increase on exposure to these conditions were also determined. Evidence for edema in the lung was determined by computing the wet-weight to dry-weight ratio.

The finding of significantly higher levels of MDA and lipofuscin in some tissues suggests that short exposure (up to 24 hours) to simulated high altitude favors

peroxidation of lipids in these tissues. Enhanced lipid peroxidation in the lung, evident after 2 hours exposure to a hypobaric atmosphere but not evident at longer exposures, may have contributed to incipient pulmonary edema.

Differences found in the levels of cytochrome P-450 suggests that the turnover of this protein in the liver of rats following exposure to simulated high altitude differed from normal.

The duration of the exposure to a hypobaric atmosphere enhanced weight loss over and above that lost by suppression of food and water.

These results suggest that various factors including the nature of the hypoxic atmosphere, the duration of the exposure, and the lack of food and water intake may alter the effect of free radicals and thus the peroxidation of lipids and proteins such as cytochrome P-450 in certain tissues. Furthermore, these results suggest that activation of free radical processes in mammals at altitude may contribute to membrane damage and thus to the etiology of altitude sickness.

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Table of Contents

Chapter — Page

I. General Intoduction 1

A. Introductory Statement..... 2

B. Some Oxygen and Hydrogen Peroxide Free Radical Reactions..... 7

C. Lipid Peroxidation..... 10

D. Historical Information..... 12

E. Suggested Causes of Altitude Sickness.... 14

F. Symptoms of Altitude Sickenss..... 15

G. References 18

II. A Comparison of Two Methods Used to Assay Lipid Peroxidation..... 20

A. Introduction..... 21

B. Methods..... 24

a. malonaldehyde (MDA)..... 25

b. lipofuscin..... 25

c. storage of tissue homogenates..... 26

C. Results..... 28

D. Discussion..... 30

E. References..... 35

III. Evidence for Action of Free Radicals in Tissues of Rats Exposed to Hypoxic Environments..... 37

A. Introduction..... 38

B. Methods..... 43

I. Animal and exposure conditions..... 43

II. Chamber design and tests..... 45

III. Chemical and physical procedures..... 49

IV. Other indices of tissue function..... 49

V. Statistical analyses..... 51

C. Results..... 52

D. Discussion..... 60

E. References..... 73

IV. Changes in Free Radical Activity in Tissues of Rats with up to 24 Hours Exposure to Simulated Altitude..... 78

A. Introduction..... 79

B. Methods..... 82

C. Results..... 85

D. Discussion..... 95

E. References..... 102

V. Conclusions and Recommendations..... 106

A. References..... 109

List of Tables

	Description	Page
Table II.I	MDA and lipofuscin (Incubation 37C)...	29
Table III.I	MDA after 2 hours exposure.....	54
Table III.II	Lipofuscin after 2 hours.....	55
Table III.III	Cytochrome P-450 after 2 hours.....	57
Table III.IV	Lung water, Hct, MetHb.....	59
Table IV.I	MDA up to 24 hours after.....	87
Table IV.II	Lipofuscin up to 24 hours after.....	88
Table IV.III	Cytochrome P-450 up to 24 hours.....	91
Table IV.IV	Lung water, Hct, MetHb.....	93

List of Figures

Description	Page
Figure I. Hypobaric chambers.....	47
Figure II. Gas-mixture chambers.....	48

Chapter I

General Introduction

A. Introductory Statement

In man, laboratory rats, and other mammals, exposure to altitudes exceeding approximately 3000m has been observed to induce injury to tissues, particularly the lung. Although the mechanism underlying the injury on exposure to such hypoxic atmospheres is uncertain it has been recognized, in rats, to resemble that produced by their being exposed to high concentrations of oxygen. The injury produced by the latter, especially by 100% oxygen, has been attributed to adverse effects of oxygen radicals (free radicals) which are increased under such conditions. The action of these highly reactive transients generated within tissues may contribute to the cellular injury evident in man and other susceptible mammals when they are exposed to high altitude, an environment in which man is frequently exposed to hypoxic conditions. The oxygen and hydrogen peroxide free radical generating reactions (details of reactions given in section B) has been recognized to be a fundamental process underlying tissue injury under many conditions.

A number of conditions have been found to activate the oxygen and hydrogen peroxide free radical mechanisms. The details of these reactions in biological systems has been reviewed by many authors (Singh, 1982; Freeman and Crapo, 1982; Marklund and Gerdin, 1983; Dormandy, 1983; Free-

man, 1984; Halliwell and Gutteridge, 1984; Jamieson et al., 1986). In addition to breathing 100% oxygen, other initiating conditions include ischemia in tissues followed by reperfusion with oxygenated blood, reactions of certain drugs with cytochrome P-450, severe exercise, withholding food and water, reactions of tissue peroxides with ions such as iron or copper, and exposure to hypoxic environments. Evidence for the latter is limited.

A reduction in the partial pressure of oxygen is a major condition affecting mammals exposed to altitude. The debilitating symptoms which often develop in man and other mammals on exposure to this environment may reflect damage to cell membranes induced by free radicals. For example, edema, in which there is increased leakage of fluid and protein to the extravascular space; proteinuria in which there is leakage of small proteins through the glomerular membrane; hematuria and hemolysis in which hemoglobin has leaked through the red cell membrane and passed through the glomerular membrane; all these symptoms suggest an altitude-induced change in membrane properties causing an increase in membrane permeability which in turn may be initiated by free radicals.

Several conditions encountered by individuals at high altitude may act to enhance the effect of free radicals or act to precipitate development of (or ag-

gravate) symptoms of mountain sickness. The most evident condition is exposure to a hypoxic atmosphere. Undertaking exercise at altitude is known to hasten development of and increase the severity of symptoms. One's capacity for exercise under such conditions is limited by the low partial pressure of oxygen so that even exercise such as climbing becomes relatively severe. These stressors are known to cause depletion of glycogen thus limiting glycolysis, the production of adenosine triphosphate (ATP) and possibly other essential cofactors discussed below.

Various underlying reactions at the cellular level may act to enhance the generation of, or interfere with the removal of free radicals when glycogen stores are reduced. One possibility is that the depletion of glycogen reduces the availability of substrates essential to maintain glycolysis, oxidative phosphorylation or other metabolic pathways that prevent free radicals from accumulating. Maintaining adequate levels of glutathione (GSH), for example, limits the accumulation of hydrogen peroxide or lipid peroxides, both potent free radical generators. In order to maintain glutathione levels reduced nicotinaminde adenine dinucleotide phosphate (NADPH), is essential for the activity of glutathione reductase. To regenerate NADPH by the pentose pathway an adequate supply of glucose is essential. According to Kappus and Sies (1981)

any stress on cells which results in depletion of substrates required to maintain adequate levels of NADH, NADPH or GSH would depress the activity of free radical scavenging enzymes. Glycogen depletion, by limiting glucose formation, tends to depress regeneration of these essential cofactors. Suppression of the desire for food is a common effect of high altitude exposure and might depress glycogen synthesis. Related to this reduction in food intake observed in humans at altitude, preliminary observations on rats indicated that during exposure to simulated high altitude for up to 24 hours, the rats neither ate nor drank.

Possible cellular reactions by which hypoxic atmospheres may activate the superoxide system (details given in section B) and enhance membrane damage develop in the mitochondria. According to Fridovich (1979), reduced levels of oxygen at the mitochondria causes components of the electron transport chain to become more reduced. Some authors(Misra and Fridovich,1982;Boveris et al.,1976; Turrens and Boveris,1980) have suggested that in such hypoxic mitochondrion electron carriers preferentially carry out single-electron transfers increasing production of free radicals. This mitochondrial activation appears to be mediated through NADH oxidase, NADPH oxidoreductase and ubisemiquinone. When these enzymes are act-

ive superoxide is generated. According to Boveris et al., (1976) utilization of ubiqinone favors the generation of free radicals thus free radical-induced membrane damage may be enhanced when these pathways are activated.

Various ischemic events in tissues at altitude may lead to increased generation of free radicals. McCord (1987) postulated that ischemic conditions, such as those observed by Whons (1986) in the retina of mountain climbers, may cause rapid conversion of xanthine dehydrogenase to xanthine oxidase so that oxidation of hypoxanthine then generates superoxide. According to Halliwell and Gutteridge (1982) when ATP is broken down and not regenerated in hypoxic tissues such as would occur at altitude, purine nucleotide degradation would occur causing hypoxanthine to accumulate. These conditions normally generate superoxide. In addition when an ischemic tissue or region of tissue is reperfused with blood containing oxygen, rapid generation of superoxide and hydrogen peroxide is likely to occur since hypoxanthine is oxidized. This ischemic-xanthine effect may also be enhanced in mammals exposed to altitude thereby increasing the possibility for tissue injury.

When free radicals such as superoxide or free radical precursors such as hydrogen peroxide or lipid peroxides accumulate in cells, peroxidation of membrane-associated particles, particularly unsaturated lipids

(details given in section C) is enhanced. The concentration in tissues of certain byproducts i.e., malonaldehyde (MDA) and lipofuscin, of this process is often used as an index of tissue damage.

B. Some Oxygen and Hydrogen Peroxide Free Radical Reactions

In review articles by Marklund and Gerdin (1983) and Halliwell and Gutteridge (1984), the following chemical reactions are proposed to be responsible for the generation of and protection against oxygen derived free radicals in biological systems.

Some free radical generating reactions:

---

1. $O_2 \xrightarrow{+e} O_2^{-\cdot} \xrightarrow{+e + 2H^+} H_2O_2 \xrightarrow{+e \ +H^+} OH^{\cdot} \xrightarrow{+e + H^+} H_2O$

oxygen — super oxide — hydrogen peroxide — hydroxyl radical — water

---

2. $R{-}\overset{H}{\underset{H}{C}}{-}H + O_2 \longrightarrow R{-}\overset{H}{\underset{H}{C^{\cdot}}} + O_2^{-\cdot} + H^+$

unsaturated lipid — oxygen — lipid radical — super oxide° — proton

---

3. $Hb(Fe^{2+})\text{-}O_2 \longleftrightarrow Hb(Fe^{3+})\text{-}O_2^{-} \longleftrightarrow Hb(Fe^{3+}) + O_2^{-\cdot}$

hemoglobin — (metHb) — superoxide

---

4. The Haber-Weiss reaction:

$$H_2O_2 + O_2^{-\cdot} \xrightarrow[Fe]{Cu} OH^{\cdot} + OH^{-} + O_2$$

hydrogen peroxide	super oxide	hydroxyl radical	hydroxyl ion	oxygen

---

5. The Fenton reaction:

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

hydrogen peroxide	iron ferrous	iron ferric	hydroxyl radical	hydroxyl ion

---

Some free radical scavenging reactions:

---

1. Action of superoxide dismutase (SOD):

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^{+} \xrightarrow{(SOD)} O_2 + H_2O_2$$

super oxide	super oxide	protons	oxygen	hydrogen peroxide

---

2. Action of catalase:

$$H_2O_2 + H_2O_2 \xrightarrow{(catalase)} 2H_2O + O_2$$

hydrogen peroxide	hydrogen peroxide	water	oxygen

---

3. Action of glutathione peroxidase (GPase):

$$H_2O_2 + 2GSH \xrightarrow{(GPase)} 2H_2O + GS\text{-}SG$$

hydrogen peroxide	glutathione (reduced)	water	glutathione (oxidized)

---

4. Action of glutathione reductase (GRase):

$$GS\text{-}SG + 2\ NADPH \xrightarrow{(GRase)} 2GSH + 2\ NADP^+$$

glutathione (oxidized)	nicotinamide adenine dinucleotide phosphate (reduced)	glutathione	(oxidized) form

---

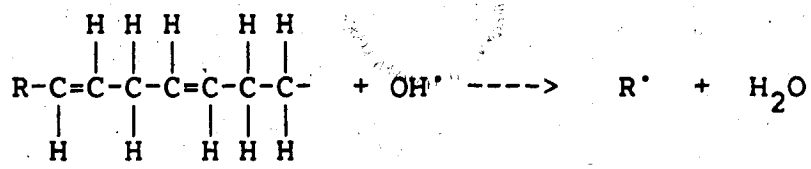
Approximately 95% of the oxygen taken in is ordinarily utilized to produce energy transformations. Under normal conditions most authors agree that a small portion of the oxygen (5%) is converted to "active" forms of oxygen or free radicals. However, under abnormal conditions, such as exposure to hypoxic atmospheres, some suggest that free radical scavenging reactions (2,3,4 above) may be depressed. If these scavenging mechanisms are depressed hydrogen peroxide would accumlate and the generation of free radicals (4,5 above) would increase. The presence of more free radicals could lead to even further propagation of lipid peroxidation.

C. Lipid Peroxidation

Reviews by Marklund and Gerdin (1983) and Halliwell and Gutteridge (1984) suggest that lipid peroxidation is an autocatalytic reaction. When the hydoxyl radical or superoxide anion interact with unsaturated fatty acids in membranes, a series of reactions involving several types of free radicals likely occur. This type of reaction (lipid peroxidation) commonly occurs as fat becomes rancid. Generally the process is considered in three stages including:

1. Initiation,

The process apppears to be enhanced when free radical scavenging mechanisms within tissues are "overloaded" or depressed and free radicals accumulate. It involves reaction of an hydroxyl radical (OH·) with part of an unsaturated membrane phospholipid as represented below:



In this the hydroxyl radical abstracts a hydrogen atom from carbon atoms adjacent to double bonds. In the process, water is formed and an "unpaired" electron is left on the carbon atom from which the hydrogen atom (containing one electron) was abstracted.

2. Propagation,

The carbon radical (a radical is any molecule with an "unpaired" electron) now a part of the membrane phospholipid, undergoes molecular rearrangement to form a conjugated diene. This molecular configuration is then susceptible to interaction with oxygen to form a membrane associated peroxy radical (one unpaired electron).

$$R^{\cdot} + O_2 \longrightarrow RO_2^{\cdot}$$

$$RO_2^{\cdot} + RH \longrightarrow ROOH + R'^{\cdot}$$

The peroxy radical may then abstract a hydrogen atom from an adjacent unsaturated membrane phospholipid to continue formation of peroxy radicals and lipid peroxides (above), or

3. Termination,

$$R^{\cdot} + \text{H-vit E} \longrightarrow R\text{-H} + \text{vit E}$$

The peroxy radical may abstract a hydrogen atom from vitamin E (an antioxidant) and become stable or react with another peroxy radical (below) and terminate the oxidative process.

$$R^{\cdot} + R^{\cdot} \longrightarrow R\text{-}R$$

The degradation of lipid peroxides (ROOH) forms a number of products including dienes, trienes, pentane, malonaldehyde and lipofuscin, a non-degradable residue. These byproducts can also impair cell function causing cell death. The occurence of these byproducts in higher than

normal concentrations has been recognized to indicate increased activity of free radicals.

D. Historical Information

People have long been aware of the suffering caused by going to high altitude although they had little knowledge of possible underlying mechanisms responsible for the symptoms. The Chinese made reference to the Greater and Lesser Headache Mountains as early as 100 A.D.; the mountains were so named because ascending them caused headache, vomiting, and dizziness. Peruvians have used the terms "puna" or "soroche" to describe mountain sickness. "Puna" refers to the high barren deserts in Peru, while "soroche" is a term describing ore bodies(lead or antimony) found at high elevations in South America. The natives blamed mountain sickness on emanations from these ores. Ravenhill (1913) while on an expedition to the high Andes region of Peru, gave detailed accounts of "normal puna"(acute mountain sickness), "cardiac puna"(pulmonary edema) and "nervous puna" (cerebral edema).

Although many general concepts regarding the causes of tissue injury in mammals exposed to hypoxic atmospheres have been proposed, the mechanism(s) at the cellular level that cause the change in cell function remain uncertain. The severity of tissue injury appears to de-

pend on the degree of hypoxia as well as on susceptibility of the particular species of mammal to hypoxia. At an altitude of 6000m, where the partial pressure of oxygen is approximately one half that at sea level, no mammals are able to adapt. Examination of organs from mammals that have succumbed to the adverse conditions at altitude has indicated that extensive tissue damage occurs, particularly in the lungs.

That the partial pressure of oxygen can adversely affect the severity of the symptoms was dramatically illustrated during early balloon flights over Paris. According to Fulton and Wilson (1966) in one of these flights, April 15, 1875, Paul Bert, a physiologist who had been studying the effects of hypobaric atmospheres realized that his colleagues had not been adequately supplied with oxygen for the flight and subsequently attempted to warn them. His advice arrived after liftoff and during the 2 hour balloon ascent to 26,300 feet two of his colleagues died. Findings today, such as the ascent of Mt. Everest by Habeler and Messner without the use of supplemental oxygen, indicate that had the balloonists ascended slowly they may have survived.

Most individuals appear vulnerable to altitude sickness although susceptibility varies. The adverse effect of hypoxia was commonly experienced during early flight of

commercial air travel. Altitude sickness was a common occurance when nonpressurized aircraft were used. This has been documented by Josephy (1962) and more recently by Gibbs-Smith (1974). A further example of the general susceptibility of men to altitude sickness was described by Singh et al.,(1965) who reported that air transport of Indian Troops from the Plains of India to the high Mountainous Regions of the Tibetan Plateau induced acute pulmonary edema in approximately 20% of the troops.

E. Suggested Causes of Altitude Sickness

Several underlying mechanisms have been proposed that could contribute to the development of symptoms associated with altitude related diseases. Singh et al., (1965) suggested antidiuresis and fluid rentention as a possible cause. Gray et al. (1975) and Sharma and Hoon (1978) postulated that abnormal platelet adhesiveness with local damage to capillaries may lead to edema formation. Hypoxia induced vasoconstriction causing an increase in shunting of blood within certain organs has resulted in the over perfusion concept developed by Hultgren et al.(1966). Houston (1976) suggested that depression of the sodium pump may lead to accumulation of sodium within cells thus drawing water into cells. Pines (1978) found hemoglobinuria in 3 climbers in Africa who developed severe acute mountain sickness. The latter ob-

servations suggest that an hypoxia-induced change in membrane structure or function may be a fundamental change underlying the symptoms of altitude illness.

F. Symptoms of Altitude Sickness

Several species of mammals have been found to be particularly sensitive to the adverse conditions at altitude. Humans and certain strains of domesticated cattle appear to be the most sensitive. A series of disabling symptoms, referred to under the general term " altitude sickness", has long been reconized to occur in those who cannot tolerate the conditions. In humans, the first symptoms are usually mild often consisting of no more than a headache, shortness of breath, fatigue and/or malaise. With intense exertion, however, or rapid ascent the symptoms typically become more severe. Additional symptoms including loss of appetite, difficulty in performing fine motor functions or simple mental tasks, nausea/vomiting, and Cheyne Stokes respiration during sleep may develop. A generalized edema is evident from swelling of loose skin of the hands and face, especially about the eyelids. In the individuals in whom these symptoms do not abate as usually occurs as acclimatization develops, pulmonary or cerebral edema may follow. The latter conditions can be fatal unless the patient is removed from the precipitating conditions. Cattle develop difficulty

in breathing which is associated with edema in the brisket, or breast region. This condition is referred to as brisket disease.

Today, with the evident increase in numbers of trekkers at high altitudes, the incidence of AMS has increased significantly and raised many questions including the need to understand the nature of the underlying cause(s) of the symptoms. Although insufficient oxygen is still recognized as the "primary" cause of AMS and altitude sickness in other mammals, the possible mechanism(s) by which hypoxia induces damage at the membrane level remains uncertain. The possibility that free radical activity may be enhanced in tissues by exposure to such hypoxic conditions and lead to membrane damage has been examined in these studies.

As part of this study an evaluation and comparison of two indices of free radical-induced lipid peroxidation was conducted in an in vitro study (details given in chap. II). Other parts of the study included an examination of the effects of conditions such as hypoxic atmospheres (details given in chap. III), the duration of the exposure to simulated high altitude (details given in chap. IV), and withholding food and water on a number of reactions that appear to be sensitive to the action of free radicals. Indices of lipid peroxidation (MDA and lipofu-

scin) in several tissues and the concentration of cytochrome P-450, a protein in the rat liver known to be sensitive to free radicals, were measured and compared.

Other indices including edema, hematocrit, and methemoglobin which are known to increase on exposure to these conditions were also determined. Evidence for edema in the lung was determined by computing the wet-weight to dry-weight ratio.

Measurement of all the above indices were made at several time intervals for up to 24 hours exposure. This period of exposure to hypoxia or 24 hours without food or water was chosen because many symptoms of acute mountain sickness often develop in susceptible individuals during this period.

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Chapter II

A Comparison of Two Indirect Methods Used To Assay Lipid Peroxidation

A. Introduction

Lipid peroxidation is generally recognized as an oxidative deterioration of fats that can lead to tissue injury. Cell membranes are recognized to be particularly vulnerable to this process once it is initiated since the double bonds of unsaturated fatty acids tend to react with free radicals thus propagating the reaction. These reactions of free radicals with membrane lipids produces many biproducts which are often measured as a means of assessing the extent of damage to tissues.

Many different methods have been used to obtain an indication of the degree of lipid oxidation of tissues. In a comprehensive review of methods Gray (1978) indicated that the method of choice is dependent upon the information required, the time available, the nature of the oxidized sample, and the test conditions. Some methods are more applicable to certain lipid components than others and can only be used to gain information about particular stages of the oxidative process (details of reactions given in Chap. I).

Although several authors recommend that more than one index of lipid peroxidation be measured when evaluating tissue damage, few studies have followed this recommendation. In this study two of the methods evaluated by Gray (1978), the chemical-thiobarbituric acid (TBA) test

and the physical-fluorescence intensity test are compared. The reaction of TBA with malonaldehyde (MDA-a byproduct of the reaction of free radcials with unsaturated fatty acids) produces a colored product, the intensity of which may be used for determining oxidative damage to tissues. Determination of MDA in tissues is the index most commonly used to indicate the extent of lipid peroxidation.

Although measurement of this index has been widely criticized, many authors (Slater,1982;Dahle et al.,1962; Halliwell,1984; and Gutteridge,1982; Halliwell and Gutteridge 1985b) agree that changes in the values for MDA may be a significant index of damage provided the same procedures are employed in the same way in each assay of an investigation. This is because the TBA assay has been found to vary with the assay conditions such as the type of acid used for development of the reaction, the temperature (of the water bath), the pH of the reacting medium and the duration of incubation. The above authors have suggested that the TBA test is a qualitative and comparative index of lipid peroxidation and not a quantitative assessment.

Other products are formed when lipids are oxidized. Many authors (Chio et al.,1963;Chio and Tappel,1969; Tappel et al.,1973;Tappel,1975,Malshet and Tappel,1973; Bidlack and Tappel,1973;Roubal,1971;and Roubal and Tappel,1966;Nair et al.,1986;) have shown that fluorescent

products, resembling "lipofuscin" are formed through the interaction of peroxidized lipids and various cellular constituents including some phospholipids, ribonuclease, proteins with reactive amino groups, protein fragments and carbonyl compunds such as MDA. These authors all have found that the development of fluorescence intensity closely parallels oxygen absorption and it can be used quantitatively to assess accumulated damage in tissue samples. They have suggested that the fluorescent method is 10 to 100 times more sensitive that the TBA assay.

In the present study two independent methods for assessing oxidation were compared in the same samples using homogenates of the heart, kidney and lung. In order to evaluate a possible relationship between MDA and lipofuscin (one of the fluorescent byproducts of lipid peroxidation) in tissue homogenates, lipid peroxidation was facilitated by incubating the samples at 37C and aliquots of the homogenate removed immediately, after 3 hours and after 24 hours incubation and analyzed for MDA and lipofuscin.

B. Methods

Six male Sprague-Dawley rats weighing 350-450g were maintained in the University vivarium. They were fed rat chow(Ralston-Purina checkers) and water ad libitum. At all times they were kept on 12 hour on, 12 hour off room light. The rats were killed in the morning(10:00 AM) and organs removed and frozen in pentane chilled in liquid nitrogen and then stored at -70C until analyzed.

Malonaldehyde (MDA): The level of MDA in tissues was determined using a modified method of Buege and Aust (1978). In this 5 %(by weight) homogenates were prepared as follows. A weighed sample (approx. 0.6g) of each frozen tissue was added to 4 ml of ice cold 1.5% KCl and homogenized with an ultrasonic homogenizing probe (Kinematic GmbH Polytron) for 20 seconds at a setting of 7 on the homogenizer. The homogenate was then diluted with additional cold KCl to give a final concentration of 5%(by wt.) of tissue. Test tubes containing the homogenate were mixed by inverting the tubes and then stored at -20C until analyzed.

The HCl-thiobarbituric acid (TBA) reagent solution used to determine MDA was prepared as follows: 2.5 ml of concentrated HCl was added carefully to 97.5 ml of distilled water and mixed gently using a magnetic stirrer. Next, 0.375 ml was removed from the beaker and 0.375 g of thiobarbituric acid (powder) added to it making 100 ml

of TBA reagent solution. To ensure dissolution of TBA powder, the beaker containing the solution was warmed to 37C on a temperature controlled magnetic stirrer.

Malonaldehyde: MDA was determined in triplicate as follows: 700 ul of a 5% tissue homogenate (w/v) was added to 300 ul of distilled water in a test tube before addition of the HCl-thiobarbituric (TBA) reagent solution. For determination of liver MDA, 900 ul of homogenate was used. For each determination, two ml of the TBA reagent was added to the sample, mixed on a vortex-type mixing device (Thermolyne Maxi- mix) then digested for 8 minutes at 95C in a water bath. At the end of the digestion period the samples were transferred to a second water bath at room temperature to cool. After cooling, 4 ml of butanol was added to the sample and the tube capped then shaken vigorously for 5 seconds. After centrifugation at 3000 rpm for 10 minutes, an aliquot of the butanol extract was transferred to a cuvette and the optical density at 532 nm (the maximum absorption for MDA) determined with a spectrophotometer (Beckman Model B). A stock solution of malonaldehyde (tetramethylacetal-TMA) was used to form a series of dilutions over the range 100nM to 800nM. The mean of 3 determinations per sample was recorded and used in statistical analyses.

Lipofuscin: Lipofuscin was estimated by a modification

the fluorescent method of Dillard and Tappell (1984). In this 500 ul of the 5% tissue homogenates (w/v) were added to 3 ml of an extraction mixture consisting of 2:1 (v:v) chloroform:methanol. To prevent further oxidation of lipids in the samples during the procedures they were mixed while under vacuum (-680 mmHg) for approx. 3 min. In this manoeuver evaporating off the chloroform served to concentrate the lipofuscin in the methanol. A further two ml of methanol was added to the test tube and the contents mixed for 5 seconds. The mixture was centrifuged (1000g for 10 minutes) and an aliquot of the supernatant transferred to a cuvette and its fluorescence determined using a fluorometer (Turner model 111). Excitation was 360 nm and emission measured at 430 nm. Results were expressed in relative fluorescence intensity units using tetraphenylbutadiene (10^{-7}mol) as a reference standard. The fluorescence intensity of 3 standard series of dilutions formed a straight line.

Storage of Tissue Homogenates

Storage temperature was found to affect lipid peroxidation of the homogenates. In order to be valid comparisons between indices had to be made on either freshly prepared homogenates or on freshly prepared homogenates that were immediately frozen and stored cold for later analysis. To ensure uniformity precisely the same proce-

dure had to be employed in each instance on homogenates that in turn had been prepared in the same manner and stored under the same conditions.

The presence of catalytic metal ions has also been recognized to effect the oxidative process in tissue homogenates. Gutteridge, (1985) and Halliwell and Gutteridge (1985a,b) have shown that certain ions enhance free radical action. One possibility is that the process of homogenization of the tissues disrupts membranes and releases potent oxidizing agents such as iron and copper ions into the medium, consequently, lipid peroxidation will likely increase on storage of the samples because of the presence of these ions.

Statistical Analyses

ANOVA with repeated measures of the results of analysis of lipid peroxidation in the same samples alternatively for MDA or lipofuscin analyses were made in order to make comparisons between means of the results. The Pearson Product-Moment Correlation Coefficient, "r" was computed to indicate possible relationships between the two indices.

C. Results

Significant increases in the concentration of MDA occurred during the first 3 hours incubation in the homogenates prepared from the 3 tissues (Table 1). During 24 hours incubation, peroxidation appeared to have continued in the heart and lung preparations. In contrast, concentration of MDA in the kidney homogenate was less i.e. MDA concentration was less following 24 hour incubation than after 3 hour.

A direct correlation ($p<.01$) was found between MDA and lipofuscin in the heart, and lung following incubation of the homogenates for up to 24 hours. The levels in the kidney homogenate did not show the same correlation. During the initial phase of peroxidation i.e., up to 3 hours incubation, a direct relationship was found, however, during the latter phase of peroxidation an inverse relationship between MDA and lipofuscin was evident ($p<.05$).

Table I. Indices of lipid peroxidation in rat tissue homogenates after incubation at 37C.

	Malonaldehyde ($nmol.L^{-1}$)			Lipofuscin (10^{-7} mol)*		
	0	+3	+24hr.	0	+3	+24hr.
Heart(6)	302 (9)	363[a] (12)	400[b] (8)	40 (1.2)	44.5 (1.3)	50.0[b] (1.1)
Kidney(6)	350 (10)	448[a] (15)	417[a] (7)	57 (1.1)	62.5 (1.3)	92.5[b] (2.0)
Lung(6)	175 (4)	212[a] (7)	226[a] (7)	10 (1.0)	10.5 (0.8)	14.1[b] (0.9)

Values = mean of 6 means, S.E.(in brackets)

(6) = number of rats killed

Tukey's HSD($p<.05$)

[a] vs initial values

[b] vs initial values and 3hr. values

* expressed relative to butadiene fluorescence(10^{-7} mol)

Discussion

A significant relationship was found between the two indices (MDA and lipofuscin) of lipid peroxidation examined in this *in vitro* study. As may be seen in Table I for the heart and lung, peroxidation apparently continues up to, at least, 24 hours incubation. In the kidney homogenate the decreased concentration of MDA found between 3 and 24 hour may be due to its conversion to lipofuscin. The overall duration of the incubation evidently had a significant effect on peroxidative processes in these tissue homogenates suggesting that product formation is time dependent.

Lipid peroxidation appears to occur in at least two phases. Since there is no significant difference in lipofuscin during the early stage of high lipid peroxidation evidenced by MDA formation, these results suggest that evidence of lipid peroxidation may be determined by early and continuing MDA production, i.e., both following 3 and 24 hours incubation while lipofuscin formation is evident during the final phase, i.e., from 24 hours onwards after incubation. These findings are in line with the interpretation of others including Bidlack and Tappel, (1973) and Dillard and Tappel, (1971) who suggested that lipofuscin is formed from the crosslinking of MDA with other cellular fragments. They further indicate that a degree of

peroxidation of lipids, i.e., production of MDA must occur before lipofuscin is formed. After 3 hours incubation it appears that MDA production has stabilized. Possibly some of the MDA is converted to the fluorescent pigment. The reduction in MDA levels found in the kidney preparation up to 24 hours incubation with a concomitant sharp rise in the concentration of lipofuscin in the same preparation tend to confirm this hypothesis. Another possibility, that Gutteridge (1985) has demonstrated, may be due to the presence of catalytic metal ions in these homogenates which can increase the generation of free radicals and thus indices of lipid peroxidation.

The correlation coefficients found in this study between MDA and lipofuscin suggests that care must be taken when formulating conclusions based on the measurement of only one index. The significant increases in lipofuscin found in all preparations in this study up to 24 hours incubation with a concomitant leveling off or decrease in the concentration of MDA suggests that lipofuscin formation occurs subsequent to the peroxidative process. Furthermore, these results indicate that if lipid peroxidation is ongoing or enhanced, inverse relationships between these indices may develop. Perhaps it is of significance that in forming such fluorescent products MDA is removed and apparently no longer indicated by the TBA

assay.

Few authors have compared two assays of lipid peroxidation determined on the same preparations. Many authors including Bidlack and Tappel(1973), Malshet and Tappel (1973), Tappel et al.,(1973), Chio et al.,(1963), Tappel, (1975), Dillard and Tappel(1971), and Gutteridge(1985) have measured one or other of these indices of lipid peroxidation, but few compared them. Dillard and Tappel (1971) determined both MDA and fluorescence intensity in rat liver mitochondrial and microsomal preparations. They found MDA levels initially increased then stabilized or decreased after 4 to 6 hours incubation. These findings resemble those of the present study for kidney homogenates. The above authors found fluorescence intensity to be maximal in their preparations at [illegible]urs at which time they terminated the incubation. They suggested that in those instances when they found a decrease in the concentration of MDA it had been taken up to form lipofuscin.

In another study using a synthetic peroxidizing system containing phosphatidyl ethanolamine and methyl arachidonate Bidlack and Tappel (1973) found thiobarbituric acid reactive substances (TBRS) reached a maximum at 10 hours incubation while fluorescence development continued and appeared to plateau after 18 hours. Of particular interest is the fact that after 10 hours incubation at 37C

they found TBRS concentration to have declined while fluorescence intensity had continued to increase. No explanation was given for what was a statistically significant decrease in TBRS. One possibility is that the decline in MDA was due to its crosslinking with amines on the phospholipid molecules forming the fluorescent product. Nair et al., (1986) have shown such a possible mode of crosslinking of proteins and amino acids with MDA.

In the present study of whole tissue homogenates, incubated for up to 24 hours at 37C, some of the changes found in the indices examined could be similar to those indicated above; i.e., MDA(a type of TBRS) production appears to increase in the heart as well as in the lung and decrease in the kidney preparations after 3 hours incubation. At the same time, fluorescence intensity increased significantly in all preparations particularly in the kidney between 3 and 24 hours suggesting that lipid peroxidation and MDA production is an ongoing process essential for the formation of lipofuscin. Evidence from this study suggests that measurement of at least two indices is preferable in assessing possible damage to tissues by lipid peroxidation since it appears that correlations between these indices may not always be direct and values for these indices, by themselves may be misleading. The duration of the incubation used in the assay appears to be

critical as the process of peroxidation is facilitated during this time.

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Chapter III

Evidence for Action of Free Radicals in Tissues of Rats Exposed to Hypoxic Atmospheres

A. Introduction

Acute exposure of man and other mammals to high altitudes apparently causes tissue injury which, if severe, is likely responsible for the development of a variety of debilitating symptoms commonly referred to as altitude sickness. The effect at the cellular level that leads to the development of the symptoms remains uncertain although hypoxia is generally recognized to underly the tissue damage. That low oxygen is likely the major factor is evident from the relief of symptoms obtained by giving supplemental oxygen. However, in some instances, this procedure was found to be not fully effective and transport of the afflicted to a lower altitude provided the only certain relief. Hackett(1980) and Houston and Dickinson(1975) have shown that descent is much more effective in ameliorating the symptoms of acute mountain sickness(AMS) than giving supplemental oxygen. These observations indicate that the increase in partial pressure of oxygen found at lower altitudes is not the only factor restoring normal tissue function. Thus, in addition to hypoxia, sudden changes in atmospheric pressure or some other factor might have a role in AMS.

Most researchers studying the effects of hypoxia often assume the physiological reactions to a hypobaric type hypoxic atmosphere (e.g. one half atmosphere) iden-

tical to those to a normobaric atmosphere containing a lower than normal fraction of oxygen (e.g. 10% oxygen in nitrogen). There have been few studies in which the two effects have been compared. In one Russell and Crook (1968) reported having found total urinary nitrogen, urea excretion, blood lactic acid, and serum urea concentrations increase linearly with the decrease in pO_2 in rats exposed to either type of hypoxic atmosphere suggesting no difference in certain metabolic functions. Levine (1988) cautions that atmospheric pressure and oxygen partial pressure may affect cell function differently, particularly in the lung.

There is some evidence that the two types of hypoxic atmosphere may affect pulmonary function differently. Bartlett and Remmers (1971) found in rats conditioned 20 days to a hypobaric atmosphere that the alveolar surface area and lung volumes were greater than in control rats kept in a normobaric atmosphere with an equivalent low partial pressure of oxygen. Recently, Levine et al., (1988) demonstrated in sheep that hypobaric conditions but not hypoxic conditions (equivalent pO_2) increased lung lymph flow. After exposing sheep to "2600m" for 2 hours they found a significant increase in lung lymph flow which they attributed to a greater surface area for fluid exchange under these conditions than that avail-

able for exchange under normobaric hypoxia. In another study Parker et al., (1981) attributed an increase found in the filtration coefficient of isolated dog lungs when ventilated with an hypoxic gas mixture to changes in vascular permeability, rather than an increased surface area for fluid exchange. Evidently exposure of mammals to different types of hypoxia affected mechanisms that regulate fluid balance across the vascular membrane differently.

Several conditions have been found to enhance free radical generation in tissues. Recently, several authors (McCord,1987;Slater,1982;Kappus and Sies,1981;Fridovich, 1979;Fridovich,1978;Misra and Fridovich,1972;Boveris et al.,1976) have suggested that hypoxic conditions in tissues may activate or prime tissues for the generation of free radicals. According to these authors production of these highly oxidizing agents which contain unpaired electrons tends to react preferentially with unsaturated lipids (details of reactions given in chap.I) and protein moieties of cell membranes often causing cell lysis or cell death. Hypoxia-induced activation of free radicals could alter membrane structure or function and might enhance fluid flow and protein leakage through membranes. An altitude-induced change in membrane function in other tissues such as the kidney may contribute to symptoms such as edema, proteinuria and hematuria experienced by man at

altitude. This enhanced free radical activity may also contribute to severe headaches commonly experienced by man at altitude. That membrane permeability is increased when free radical action has been induced has been shown by some authors including Halliwell and Gutteridge(1984), Repine and Tate(1983), Deneke and Farburg(1980) and Ohmori et al., (1978). Evidence indicating that exposure to hypoxic environments may enhance free radical activity in tissues of mammals, however,is limited.

In the present study the possibility that environmental hypoxia might activate free radical-mediated activity in tissues of rats was examined and the effect of two types of hypoxia compared. Hypoxic conditions equivalent to an altitude of 6000m were used as this type of exposure commonly induces severe symptoms of altitude sickness in man and other mammals. To assess possible lipid peroxidation in tissues of rats after exposure to different types of hypoxic atmospheres the concentration of malonaldehyde (MDA) and lipofuscin (byproducts of free radical-induced oxidation) were determined in several tissues. In addition,the concentration of a hemeprotein, cytochrome P-450, which some authors (Roubal,1971;Levine,1982; Levin et al.,1972 and Svingen et al.,1979) have shown to be degraded by the action of free radicals was also determined.

Other indices which have been reported to increase on exposure to hypoxic atmospheres were also measured. These included hematocrit and methemoglobin. The tendency of animals developing pulmonary edema, a common type of altitude sickness in man, was evaluated by determining the wet weight/dry weight ratio of this organ.

B. Methods

I. Animal and Exposure Conditions

Groups of 10 male Sprague-Dawley rats weighing 350-450g were exposed to either normal or hypoxic atmospheres in separate chambers described below. For the hypobaric exposure an atmospheric pressure equivalent to 355 mmHg (hypobaric-hypoxia, pO_2, 70 mmHg), equivalent to an altitude of approximately 6000m, was maintained in the hypobaric chambers using a pressure/vacuum pump(Gast-1/6 hp pressure/vacuum to 27" Hg) and a regulator (negative pressure regulator, Matheson model 3491). The hypobaric pressure in individual chambers was checked on occasion by attaching a mercury manometer to the repressurizing ports. In order to provide a normobaric atmosphere with a partial pressure equivalent to that of the hypobaric atmosphere described above, a gas mixture containing 10% oxygen and 90% nitrogen(normobaric-hypoxia, pO_2, 70 mmHg) was passed through the chambers (flow rate=5.0 $lmin^{-1}$). Samples of air within the chambers were drawn through the repressurizing ports into a 50cc syringe during the exposure condition and the concentration of carbon dioxide in the samples determined by passing them through a carbon dioxide (Capnograph Godart type KK) analyzer. These "checks" indicated that during exposure to both types of hypoxic atmospheres the carbon dioxide concentration was less than

0.5% indicating that flow through the system provided adequate ventilation. Prior to the experiments, the rats were maintained in shoe-box type cages (6 rats/cage) in the University vivarium. They were fed rat chow(Ralston-Purina checkers) and water ad libitum. At all times they were kept on 12 hour on, 12 hour off room light. The rats were then transferred in their cages to the laboratory where they were held under similar conditions. When used the rats weighed 350-450g.

Groups of 10 male rats were weighed and then exposed 5 at a time in separate chambers (described below) for 2 hours to either ambient air(controls), to a gas-mixture (normobaric-hypoxia) or to a decreased pressure(hypobaric-hypoxia). No food or water was provided in the chamber. After the exposure they were weighed and killed for tissue analysis as described below.

To evaluate the effects of withholding food and water, and/or other effects on rats such as their being placed in a new environment (the chamber), an additional group(10 normals), which had access to food and water ad libitum, were taken directly from their shoe-box cages weighed, killed and tissues stored for later analysis. In keeping with the findings of Conlee et al., (1976) the rats were killed at approximately the same time each morning(10:00 AM), a time which they found minimized the

effects of diurnal variation on glycogen stores.

The general procedure followed for preparation of tissues was as follows. On removal from the chamber the rat was reweighed, anesthetized with ether then the abdominal wall sectioned exposing the diaphragm and beating heart. This procedure facilitated cardiac puncture. The blood obtained was stored on ice in a refrigerator while various organs were removed. The lungs were blotted dry and immediately frozen in pentane chilled in liquid nitrogen (cold pentane) and stored at -70C for later analysis. The liver was blotted dry, weighed, and washed in ice-cold saline as were the heart and kidneys. The heart and kidney were then cut in half (longitudinally) and the liver lobes cut into pieces weighing approximately 2 g each. After washing in ice cold saline, the tissue samples were frozen in cold pentane and stored in vials at -70C, until analyzed for MDA and lipofuscin. Hematocrits and methemoglobin concentrations were then determined on samples of the freshly drawn blood.

II. Chamber Design and Tests

To provide the desired experimental conditions, a system of chambers, each holding one rat, was designed. This was developed (details given in Fig.I and II) so that one rat could be removed from the particular atmosphere leaving the remaining rats in the experimental at-

mosphere. One chamber could be opened by closing "isolation" clamps on air lines then opening the repressurizing port slightly allowing ambient air to enter slowly.

Before this chamber system was developed a number of preliminary tests were conducted on rats using a different chamber consisting of a heavy aluminum box which was large enough to hold two of the normal "shoe-box" cages with water jars, food and each containing 4 rats. Use of this provided some indication of how the rats would react to the stress of hypobaric conditions. One observation of three such hypobaric trials was that at "6000m" they neither ate nor drank for up to 24 hours. Subsequent hypoxic trials, therefore, were conducted without food or water at room temperature(22C).

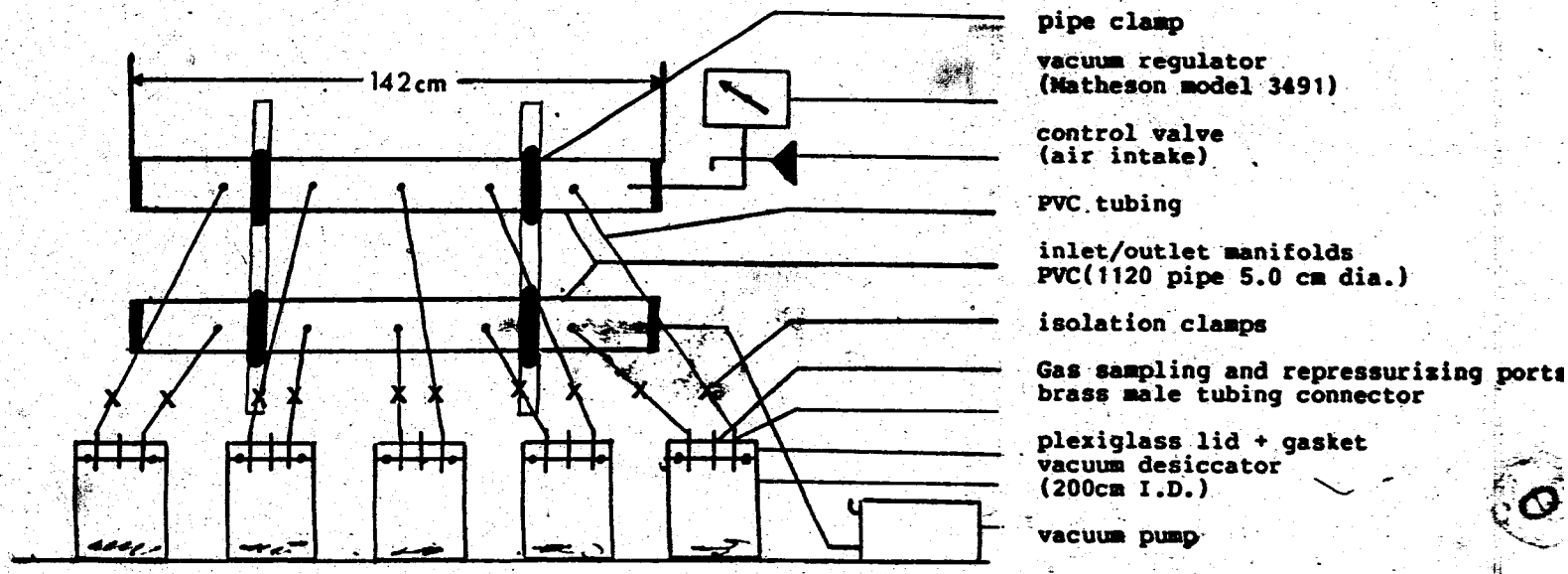

Figure 1. Hypobaric chambers used to expose rats to a pressure equivalent to 355 mmHg. With the dual manifold system, one rat could be returned to ambient conditions while the others were maintained at hypobaric pressures using a vacuum pump.

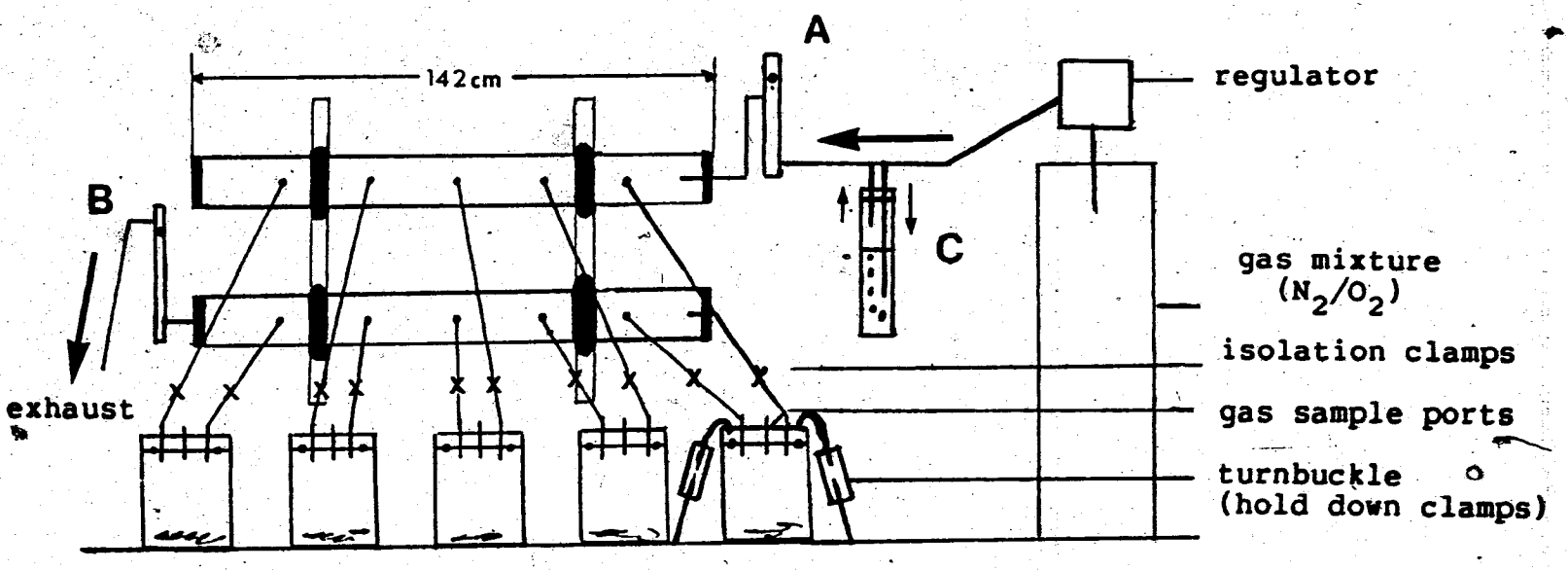

Figure 2. Hypoxic chambers used to expose rats to a gas mixture containing 90% nitrogen and 10% oxygen. The lids (12mm plexiglass) were secured on the desiccators with adjustable clamps. The gas flow (~5.0 l/min.) was regulated at "A" and monitored at "B" with Matheson model 603 variable gas meters to ascertain that the system was airtight. The gas was bubbled through water at "C" to add humidity.

III. Chemical and Physical Procedures

Malonaldehyde (MDA) in tissue homogenates was determined using a modified method of Buege and Aust (1978), (details described in chap. II). In principle the MDA reacts with an HCl-thiobarbituric acid (TBA) reagent solution yielding the colored product which was assessed spectrophotometrically.

Lipofuscin was determined using a modification of the method developed by Dillard and Tappell (1984), (details are described in chap.II). In this, the fluorescent pigment was extracted from the tissue homogenate using a chloroform:methanol mixture and the relative fluorescence intensity read using a spectrofluorometer.

IV. Other Indices of Tissue Function

Methemoglobin: The procedure for determining if methemoglobin was present was that used by the Clinical Sciences Department of the University of Alberta (unpublished). For this 100 ul of whole blood was added to 10 ml of distilled water and the contents mixed by inversion of the test tube to ensure hemolysis of the cells. The mixture was then centrifuged (1000g) for ten minutes to remove cell debris following which an aliquot of the supernatant was transferred to a cuvette and the absorbance read at 630nm in a spectrophotometer (Pye-Unicam SP 1800) to determine if an absorption peak, corres-

ponding to that for methemoglobin, was present. Disappearance of the absorption peak following the addition of 2 drops of 33% (v/v) ammonia, confirmed the presence of methemoglobin.

Preparation of Liver Microsomes and Determination of P-450: The microsomal fraction was prepared by the method described by Sladek and Mannering (1966). For this, a weighed sample (approx.1g) of frozen liver was homogenized in 3 ml of ice cold KCl(1.15%) to give a final contration of 25% of liver. The homogenate was then centrifuged in 50 ml polypropylene centrifuge tubes in a high speed refrigerated centrifuge (MSE'Superspeed'50) at 1-4C at 12,000g for 25 minutes to remove debris, nuclei and mitochondria. Following this an aliquot of the supernatant, which still contained the microsomal fraction, was centrifuged at higher speed in a 10/10 ml angle head at 78,000g for 60 minutes at a temperature of 4C. The supernatant was decanted, the pellet formed was rinsed with ice-cold 1.15% KCl and then resuspended using a homogenizer in an equal volume of the KCl. To this microsomal suspension 6.0 ml of a 0.2M phosphate buffer, pH 7.4, was added and a few crystals of sodium dithionite added to reduce the haemproteins present. The test tube was sealed with Parafilm then contents mixed by inverting the tube

2-3 times. Aliquots (3 ml) were then transferred to each of two cuvettes. Carbon monoxide (100%) was then bubbled through the contents of one cuvette for 30 seconds to reduce the methemoglobin formed. The second cuvette containing the same reagents and treated in the same manner except for the addition of carbon monoxide served as reference. The difference in absorption spectra between the sample and reference cuvettes was determined between 550 and 400 nm using a dual beam spectrophotometer (Pye-Unicam SP 1800). The cytochrome P-450 present was proportional to the absorbance at 450 nm less the absorbance indicated at 490 nm. The P-450 content was calculated using the molar extinction coefficient of 91 $cm^{-1}mmol^{-1}$ as determined by Omura and Sato (1964).

Evidence for Pulmonary Edema: One of the lung lobes from each rat was weighed and then dried in the oven at 90C for 12 hours and reweighed. The wet weight to dry weight ratios were compared. The water content (%) of the sample of lung tissue (frozen weight-dry weight/frozen weight x 100) was also calculated.

V. Statistical Analyses

Students' t test was used in making comparisons between means. The Pearson Product-Moment Correlation Coefficient,"r" was computed to suggest possible relationships between some of the indices measured.

C. Results

I. The effect of withholding food and water on lipid peroxidation and on the water content of the lung.

Comparing values for the normals with the controls reveals that withholding food and water from rats for 2 hours had little or no effect on the concentration of MDA, lipofuscin in tissues or on the water content of the lung.

II. The effect of hypoxia and withholding food and water on lipid peroxidation in tissues of rats.

Exposure of rats to normobaric-hypoxia caused a significant ($p<.05$) decrease in the concentration of MDA in the lungs, kidneys and liver but had no apparent effect on levels in the heart (Table I). It caused a decrease in lipofuscin only in the kidneys (Table II). At the same time, lipofuscin intensity was found to increase significantly ($p<.05$) in the liver (Table II).

In contrast to the decrease in MDA concentration found in most tissues of rats exposed to normobaric-hypoxia, exposure to reduced pressure (hypobaric vs controls, Table I) for 2 hours caused a significant increase ($p<.05$) in MDA in the lungs and kidneys. A significant ($p<.05$) reduction in MDA, however, was found in the heart and liver (Table I), similar to that observed in rats exposed to normobaric-hypoxia. Fluorescence due to lipofuscin decreased ($p<.05$) in the lung and increased significantly ($p<.05$)

in the heart of rats exposed to hypobaric-hypoxia.

Table I. Malonaldehyde concentration in some tissues of rats after 2 hours in hypoxic atmospheres.

Group \ Index	pO_2/pN_2	Pr. atm	Malonaldehyde (nmol.L^{-1}) Lung	Kidney	Heart	Liver
Normals (10)	145/565	1.0	184 (7)	352 (10)	302 (9)	595 (28)
Control	145/565	1.0	190 (4)	363 (11)	240 (21)	559 (17)
Normobaric-Hypoxia	70/640	1.0	123[a] (7)	231[a] (10)	296 (17)	255[a] (5)
Hypobaric-Hypoxia	70/285	0.5	231[b] (7)	470[b] (8)	161[b] (17)	470[b] (15)

Values = mean of 10 means, S.E. (in brackets)

(10) = number of rats per group

Student's t test
$p<.05$

[a] sign. difference vs other groups same column

[b] sign. difference vs normobaric-hypoxia and other groups same column

Table II. Lipofuscin fluorescence intensity in tissues of rats after 2 hours in hypoxic atmospheres.

Group (Index)	pO_2/pN_2	ATM	Lipofuscin Intensity* Lung	Kidney	Heart	Liver
Normals(10)	145/565	1.0	12 (.80)	78 (3.1)	44 (1.1)	71 (1.6)
Control	145/565	1.0	14 (.80)	76 (1.3)	42 (1.3)	79 (2.7)
Normobaric-Hypoxia	70/640	1.0	15 (.80)	67[a] (1.1)	45 (2.0)	95[a] (3.1)
Hypobaric-Hypoxia	70/285	0.5	9[b] (.40)	75 (2.4)	52[b] (1.2)	67 (1.5)

Values= mean of 10 means, S.E(in brackets)

(10)= number of rats per group

* expressed relative to butadiene fluorescence (10^{-7} mol)

Student's t test
$p<.05$

[a] sign. difference vs other groups same column

[b] sign. difference vs normobaric-hypoxia and other groups same column

III. The effect of withholding food and water on cytochrome P-450.

A significant ($p<.05$) increase in the concentration of P-450, similar to that found in rats exposed to hypobaric-hypoxia but in contrast to the decrease in P-450 found in the liver of rats exposed to normobaric-hypoxia was found in the liver of rats deprived of food and water (Table III). The mean weight loss in the rats deprived of food and water for 2 hours was 8g.

IV. The effect of hypoxia and withholding food and water on cytochrome P-450 of rats.

A reduction in the concentration of cytochrome P-450 (Table III), in contrast to the increase found in rats exposed to hypobaric-hypoxia occurred in the liver of rats after exposure to normobaric hypoxia. Rats exposed to normobaric-hypoxia for 2 hour lost slightly less weight than that lost by the rats exposed to hypobaric conditions (9g vs 12.8g). However, a significant ($p<.05$) reduction in liver weight was found after exposure to normobaric-hypoxia compared to normals, controls and hypobaric rats.

When compared to controls, exposure of rats to hypobaric-hypoxia had no significant effect on the concentration of cytochrome P-450 in the liver (Table III). No significant difference in liver weight was found (Table III) although mean weight loss was greater (12.8g vs 8g).

Table III. Liver cytochrome P-450 and weight loss in rats after 2 hours exposure to hypoxic atmospheres.

Group (Index)	pO_2/pN_2	ATM	Wt. loss (g)	Liver wt. (%FBW*)	Cytochrome P-450 nmol/gm liver
Normals(10)	145/565	1.0	0 (0)	4.27 (.12)	20.8[a] (2.7)
Control	145/565	1.0	8 (1.5)	4.29 (.05)	33.7 (1.8)
Normobaric-Hypoxia	70/640	1.0	9 (1.6)	4.00[a] (.07)	16.7[a] (2.9)
Hypobaric-Hypoxia	70/285	0.5	12.8 (1.5)	4.29 (.10)	32.8 (2.7)

Values= mean of 10 rats, S.E.(in brackets)

(10)= number of rats per group

* FBW=final body weight

Student's t test
$p<.05$

[a] sign. difference vs other groups same column

V. The effect of hypoxia and withholding food and water on the lung water content of rats

Exposure of rats to normobaric-hypoxia caused a 7% ($p<.05$) increase in the wet weight to dry weight ratio (Table IV). A significant increase (6%, $p<.05$) in hematocrit was also found after this type of exposure. Methemoglobin concentration was not affected by the exposure.

In contrast to the slight increase in the wet/dry ratio found in rats exposed to normobaric-hypoxia, exposure to hypobaric-hypoxia caused a 29%($p<.05$) increase in this index. Hematocrit also increased 4% ($p<.05$) while methemoglobin concentration in rats appeared to be unaffected by exposure to the reduced pressure (Table IV).

Table IV. Lung water of rats after 2 hours in an hypoxic atmosphere.

Group \ Index	pO_2/pN_2	ATM	Wet/Dry (ratio)	Lung water (%)	Hct. (%)	MetHb.* (Abs.)
Normals(10)	145/565	1.0	3.9[a] (.11)	74[a] (.71)	46[a] (.7)	.39 (.01)
Control	145/565	1.0	4.2 (.12)	76 (.63)	47 (1.)	.39 (.01)
Normobaric-Hypoxia	70/640	1.0	4.5 (.14)	78 (.72)	50 (.4)	.40 (.01)
Hypobaric-Hypoxia	70/285	0.5	5.4[b] (.26)	82 (.89)	49 (.4)	.41 (.01)

Values= mean of 10 means, S.E.(in brackets) for Hct. and MetHb.
mean of 10 rats, S.E.(in brackets) for wet/dry and lung water

(10)= number of rats per group

* qualitative assay

Student's t test
$p<.05$

[a] sign. difference vs other groups same column except control

[b] indicates significant difference vs normobaric-hypoxia and other groups same column

Discussion

The findings, that the concentration of MDA in the lungs and kidneys and that of lipofuscin in the liver and heart(Table I,II) increased suggests that free radical activity may have been enhanced in some tissues by 2 hours exposure to hypoxic atmospheres. The decrease in the concentration of P-450 (Table III,normobaric-hypoxia) found in the liver also supports this suggestion. However, these are indirect assessments of enhanced free radical action. Free radicals were not measured directly (e.g. as could be by assessing their characteristic electron spin resonance). However, oxygen radicals or other species of free radicals might have initiated the processes leading to changes in these indices.

The results also suggest that some other factor or factors, in addition to hypoxia per se, may contribute to the differences in the activity of free radicals in tissues of rats exposed to these environments. The most apparent difference between the normobaric-hypoxia and hypobaric-hypoxia is in the partial pressure of nitrogen (pN_2) which is lower in the hypobaric condition (285 mmHg) and higher in the normobaric-hypoxia condition (640 mmHg), approx. 70 mmHg above normoxia. Possible means by which this gas, which is usually considered to be a nonreactive diluent at such pressures, may alter processes such as li-

pid peroxidation or protein degradation, if indeed this is possible, is open to question. A further condition contributing to the responses might be in the difference in air pressure itself. The hypobaric atmosphere may be expected to be initially more stressful to the exposed animal.

The greater increase found in the wet/dry ratio (Table IV) of the lungs of rats exposed to hypobaric--hypoxia than that found in the rats exposed to normobaric-hypoxia suggests that the reduced pressure may have induced a greater increase in membrane permeability with more water accumulation evident in the rats exposed to decreased ambient pressure.

The rapid increases found in water content of the lung and indices of lipid peroxidation in certain tissues following hypobaric-hypoxic exposure are in line with the suggestion that the responses may contribute to development of injury often associated with altitude sickness in man and other mammals.

The variability in indices of lipid peroxidation assayed in various tissues of rats in this study suggests that the effect of each type of hypoxia on these tissues may differ. The cause of the differences in the concentration of MDA and lipofuscin in some tissues and not in others is not evident. Most authors indicate that when these indices increase, relative to normal values, tissue damage

is likely to be enhanced. The results from the present study which show increases in the concentration of MDA and lipofuscin in some tissues but not others suggests that those showing increased concentrations may have suffered some degree of damage by the exposure to reduced ambient pressure or normobaric-hypoxia respectively(Table I,II). Perhaps, pN_2 affected the interaction of enzymes with substrates or other membrane associated particles causing membrane damage. This is suggested by the findings of Sushella and Ramarsarma (1973) who found that liver mitochondrial succinate dehydrogenase was activated by ubiqinone in rats exposed to hypobaric atmospheres whereas, under normobaric-hypoxia (gas-mixture at same pO_2) other substrates were preferred. According to Boveris et al., (1976) utilization of ubiqinone favors the generation of free radicals thus lipid peroxidation and membrane injury may be enhanced when this pathway is active.

A clear relationship between MDA concentration and lipofuscin intensity in the different organs assayed in the present study was not evident. One possibility, pointed out by Freeman (1984), which may contribute to the different values found for MDA and lipofuscin between similar tissues and different organs, reflected differences in free radical producing and in scavenging mechanisms of tissues. The latter author contended that different

enzyme activities combined with availability of some co-factors, substrate concentration or oxygen tension can favor free radical production in excess of basal rates. The variation found in the present study in values for MDA and lipofuscin in the same tissues from different groups of rats and between organs within rats indicates that the effect of the two types of hypoxic atmospheres on factors regulating lipid peroxidation in various tissues is not consistent.

Different rates of conversion of MDA to lipofuscin in tissues may have contributed to the variation in the values seen. The increase in the levels of MDA in the lungs and kidneys of rats after 2 hours exposure to hypobaric conditions but a decrease in this index in the same organs after 2 hours exposure to normobaric-hypoxia suggests that metabolism of MDA or its conversion to lipofuscin may have been ongoing for at least 2 hours in the latter group. Since the pO_2 (70 mmHg) was the same in the two atmospheres, these results suggest that oxygen tension was not solely responsible for this difference and that some other factor may have caused the apparent increase in peroxidation in both groups of rats. Although MDA levels were found to be significantly lower than normal in the lung, kidney, and liver of the rats exposed to normobaric-hypoxia, the fluorescence intensity due to li-

pofuscin in the lungs and livers at the same time was found to have increased suggesting that conversion rates to this product may have differed in the different hypoxic atmospheres. The decreased levels of lipofuscin found in the kidneys of the rats exposed to the normobaric-hypoxia suggests that this material had been excreted. According to some authors (Tappel,1980;Dillard and Tappel,1984;and Gutteridge,1985;Roubal and Tappel,1966) the formation of lipofuscin is directly related to the concentration of MDA; the availability of reactive amino groups; the concentration of other oxidation products; and on the length of time these products have to interact. The apparent nonuniform production of MDA and lipofuscin in tissues of rats exposed to different hypoxic atmospheres supports this hypothesis.

Other factors have been suggested to affect levels of these products of peroxidation in tissues. Some authors (Iyer,1985;Rader et al.,1985 and Hashimoto et al.,1962) have shown that certain metabolites in cells appear to protect them from free radical-mediated injury. Adequate GSH, NADH, and NADPH appear to be essential in protecting tissues against free radical action. Iyer et al., (1985) suggested that in hypoxic environments, similar to those used in this study (pO_2,70 mmHg) reduced oxidation of glucose via the pentose shunt during exposure to

the hypoxic atmosphere tends to reduce regeneration of cofactors like NADPH. These cofactors were not investigated in this study. However, Reed and Pace(1980) found the concentrations of these cofactors in the liver decreased in rats after they had been exposed to "3800m" for up to 3 hours.

The lower levels of MDA in the heart and liver may reflect the greater capacity of these organs to metabolize MDA than the either lung or kidneys. These organs more readily metabolize lactate than do either lung or kidneys and thus may be able to maintain adequate pools of GSH, NADH, and NADPH. This is suggested by the findings of Felig(1965) who found that the survival rate of rats exposed to an atmosphere containing 95% oxygen was increased by feeding them sodium lactate. Exposure of rats to this type of atmosphere is known to enhance free radical formation and membrane damage. He suggested that the increased survival rate was due to enhanced generation of NADH. The decreased concentration of MDA found in the liver of rats (Table I) in the present study may have been due to its rapid metabolism by this organ. This suggestion is based upon the conclusion of Placer (1965) and Draper et al.,(1986) who demonstrated that MDA is rapidly metabolized by the liver. Since the metabolism of MDA and the concentration of reactive amino groups that crosslink with

MDA to form lipofuscin is likely to differ between tissues, the concentration of these biproducts of lipid peroxidation may be quite different in different tissues.

The catabolism of adenosine triphosophate (ATP) to hypoxanthine (details in chap.I) can affect the levels of free radicals in tissues and thus the concentration of MDA and lipofuscin. Activation of xanthine oxidase by hypoxanthine during exposure to hypoxia may increase the production of superoxide and enhance lipid peroxidation in tissues and thus the concentration of MDA and lipofuscin.

Another factor which may effect lipid peroxidation in tissues is the concentration of glucose in the perfusing fluid. Hearse et al.,(1976) and Rinette et al., (1984) have shown that adequate glucose, under some in vitro conditions, may be essential to minimize free radical-mediated cell injury. Depletion of glucose was likely not a factor in this study however since a two hour period without food and water will not drastically reduce the concentration of blood glucose in intact animals. Since the concentrations of MDA and lipofuscin in the control rats were not different from the normals (Table I, II) this suggests that glucose concentration was adequate and that withholding food and water for 2 hours was likely not a factor affecting peroxidation in tissues.

The concentration of certain antioxidants such as vitamin E or C in membranes and cells may be critical in protecting the cells from free radical attack. Exposure to hypoxia, by altering the interaction of these antioxidants with free radicals may affect lipid peroxidation in tissues and thus the levels of MDA and lipofuscin.

The processes which function to determine the concentration of P-450 in the liver are not fully understood. Recent findings of several authors (Bast and Haenen,1984;Quinn and Payne,1984;Levine,1982;Jacobson et al., 1973;Levin et al.,1972;Svingen et al.,1979) have indicated that the reaction of P-450 with a number of endogenous and exogenous substrates generates free radicals which in turn may contribute to enhanced lipid peroxidation. These researchers demonstrated that cytochrome P-450 may function in the propagation of lipid peroxidation and that free radicals so formed may tend to degrade this hemeprotein itself. Finding a decrease in the concentration of P-450 in the liver of rats exposed to normobaric-hypoxia is in line with this hypothesis. The reduction in the concentration of P-450 (Table III) found in the liver of rats exposed to normobaric-hypoxia(pO_2/pN_2, 70/640) and not in the hypobaric group (pO_2pN_2, 70/285) points to other factors and not the low partial pressure of oxygen alone as init-

iating the process leading to the apparent decrease in P-450. This may be due to activation of free radicals which in turn caused destruction of this protein. In the rats exposed to hypoxia for 2 hours, P-450 concentration was found to correlate with that of MDA ($r = .59, p < .01$) and with that of lipofuscin ($r = -.46, p < .05$). The mechanisms inducing this apparent relationship between these indices of lipid peroxidation to that of liver cytochrome P-450 is not clear from this study. One possibility is that during the initial period of lipid peroxidation, P-450 is released from the membrane resulting in increased values. According to Wolff et al.,(1986) Levine et al.,(1982) and Svingen et al.,(1979) enhanced destruction of P-450 and other proteins also may occur when depletion of cellular reductants such as NADH, NADPH, or GSH occurs. Changes in the levels of these cofactors, induced by withholding food and water or exposure to hypoxic atmospheres may have affected lipid peroxidation and protein degradation in this organ.

Rats may be sensitive to the stress of being in a new environment such as being transferred to a new cage and to being without food and water. After 2 hours of no food and water, a 50% greater concentration of P-450 was found in the liver (Table III). This suggests that the stress imposed on animals when they were placed in the

chambers was enhanced and may have facilitated the synthesis and or release of P-450 from microsomal membranes. This is not in keeping with the findings of Sadano and Omura(1982) or Kumar et al.,(1980) who found that the normal turnover of P-450 is at least 12 hours. Shiraki and Guengerich (1984) concluded that levels of microsomal enzymes in the liver are primarily a function of changes in the rates of protein synthesis as opposed to rates of degradation.

The synthesis of P-450 maybe enhanced by exposure to hypoxic atmospheres. This was shown in mice by Longmuir et al.,(1977) and coworkers(Rowe and Longmuir,1979; Longmuir and Pashko,1977). They, and Knoblauch et al.(1981), suggest that P-450 could act as an oxygen carrier and the increased synthesis on exposure to low pO_2 would enhance this function. The results of this study appear contrary to their findings in that P-450 concentration increased significantly in rats by withholding food and water alone and/or the stress of being placed in a new cage.

The increase in water content of lung tissue (Table III) during the two hours exposure to hypoxia suggests incipient edema developed in this organ. The rats exposed to hypobaric-hypoxia however, retained more water in the lungs than those exposed to normobaric-hypoxia. This suggests that the stress of the two conditions was not

identical, and some aspect of the hypobaric condition caused greater fluid to accumulate in the lungs. One possibility may be that the reduction in pN_2 enhanced free radical activity in the lung and caused an increase in permeability of the vascular membrane. The concomitant increase in the concentration of MDA (Table I) and water in the lungs (Table IV) of rats exposed to hypobaric conditions support a conclusion that a pressure-induced increase in lipid peroxidation of this organ occurred which in turn may have increased vascular permeability in the lung and contributed to water retention.

An increase in pulmonary arterial pressure has also been observed in man and in domestic animals at altitude. This has been implicated as a cause of pulmonary edema in these species. That such an increase occurs in rats in the present study and might have contributed to the fluid accumulation observed in these lungs cannot be ruled out. Levine et al., (1988) found the increase in pulmonary arterial pressure to be similar in sheep exposed to hypobaric-hypoxia as when exposed to an equivalent normobaric hypoxia. The lymph flow, however, was significantly greater when they were exposed to reduced pressure. The present results are in keeping with the suggestion that in rats exposed to hypobaric conditions the vascular membrane may have become more permeable due to lipid peroxidation

which in turn may enhance fluid accumulation in the lungs. Levine et al.,(1988) did not measure MDA in their study however attributed an increased lymph flow found in lungs of sheep exposed to hypobaric conditions to a greater surface area for fluid exchange.

Several mechanisms contribute to the increase in hematocrit observed in rats exposed to both types of hypoxic atmospheres (Table IV). The increase may have been caused by water loss which in turn would depend on the magnitude of the hyperventilatory responses elicited. Exposure to the hypoxic conditions may also have acted through some other mechanism such as depression of the activity of the sodium pump in the red cell. This would cause intracellular water to accumulate, swelling the cells, thus producing a mean cell volume increase and an increase in hematocrit.

Brief exposure of rats to the types of hypoxia described appeared to have had no effect on formation of methemoglobin. This lack of formation of methemoglobin under the conditions studied suggests that the antioxidant mechanisms of the red cell, including various cofactors, were adequate to prevent the formation of methemoglobin and superoxide.

In conclusion, findings of this study suggest that increases in the water content of the lung, which may be

related to the action of free radicals, are greater in rats exposed to hypobaric atmospheres that in those exposed to an equivalent normobaric-hypoxia. These changes appear to occur immediately becoming evident with 2 hours exposure. The magnitude and nature of these reactions which also appear to affect the concentration of cytochrome P-450 in the liver, may differ with the nature of the hypoxia and other possible stresses.

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Chapter IV[a]

Changes in Free Radical Activity in Tissues of Rats With Up[illegible] 24 Hours Exposure to Hypobaric-Hypoxia

[a] material herein includes values for normals, control and hypobaric rats from chapter III

A. Introduction

As indicated previously (chapter III) injury to tissues by oxygen radicals (free radicals) may be enhanced in laboratory rats by exposing the animals to simulated high altitude. That a major process in the injury caused by free radicals appears to be the oxidation of unsaturated membrane lipids has been postulated. Activation of this process (lipid peroxidation) in tissues results in the elevation of several products of lipid peroxidation including malonaldehyde, lipofuscin, pentane, ethane and dienes. Several authors (Tappel, 1975:Chio et al., 1963;Chio and Tappel, 1967;Dillard and Tappel, 1971;Halliwell and Gutteridge, 1985a, b;Machlin and Bendich, 1987;Fridovich, 1979; Slater, 1982;Cross et al., 1987;Fridovich, 1978;Gutteridge, 1982;Dillard and Tappel, 1984;Roubal, 1971;Kim and LaBella, 1987) have suggested that assay of certain of these products such as malonaldehyde and/or lipofuscin in tissues can serve as an index of the extent of tissue injury.

According to some authors (Dillard and Tappel, 1971; Bidlack and Tappel, 1973;Halliwell, 1987) the process of lipid peroxidation occurs in several stages. This was also discussed in chapter II. In the initial stages lipid peroxides and dienes are produced which may then react further with oxygen and eventually, produce malonaldehyde (MDA). After appreciable oxidation has taken place the

second or propagating phase, during which oxidation continues and the crosslinking of MDA with proteins or membrane associated particles occurs producing lipofuscin which accumulates. The latter process apparently continues while lipid peroxidation subsides. Evidently this process in tissues depends on numerous factors including the type and concentration of unsaturated fatty acids in membranes, the proximity of catalytic metal-protein complexes containing iron or copper, the tissue pO_2, the nature and effectiveness of free radical producing and scavenging systems and on the concentration of antioxidants including vitamin E or C. Enhancement, termination and/or abatement of this action in tissues, therefore, may occur quickly or take time, depending on the complex interaction of these numerous mediators.

The variation in the time for symptoms of altitude sickness to develop or to abate, in some instances, hours or days may be related to the fact that the complex process of lipid peroxidation in tissues occurs in several stages. Some symptoms of altitude sickness ameliorate with rest or descent suggesting that ongoing metabolism within cells can be adequate to terminate or reverse the process causing the injury. In others, remaining at altitude may result in the symptoms becoming more severe suggesting that the extended stay enhanced some mechan-

ism within cells causing the symptoms to persist. Duration of the exposure to altitude therefore, can apparently affect mechanisms within cells that determine the extent of tissue injury and, the severity of the symptoms. To suggest that the unpredictable nature and development of symptoms of altitude sickness may be related to peroxidative processes in tissues is tenuous, however, the developmental stages of the two conditions appear common to both. Previous studies (Chap.III) have suggested that short exposure (2 hours) to simulated high altitude can activate free radical induced changes in cer[redacted]issues including the lung which results in retention of more water.

In the present study laboratory rats were exposed to simulated high altitude for up to 24 hours to determine possible changes in free radical action during this time.

B. Methods

Groups of 10 male Sprague-Dawley rats (350-450g) were exposed 5 at a time in separate chambers (details given in chap.III) for 2, 6, 12, or 24 hours to either ambient air (controls) or to a decreased pressure equivalent to an altitude of 6000m (hypobaric). After the exposure the rats were killed and tissue analyzed. As rats had been found previously not to eat or drink during 24 hours exposure to such hypobaric conditions, no food or water was provided in the chambers.

In order to evaluate the effects of withholding food and water, and/or other effects on rats such as their being placed in a new environment (the chamber), an additional group (normals), which had access to food and water ad libitum, were taken directly from their shoe-box cages, weighed and killed.

The general routine was such that the control rats were in the chambers under ambient conditions for the same duration as the rats that were exposed to simulated high altitude. Thus, for example, on separate days, 5 control rats were killed after 2 hours exposure to ambient conditions and compared with 5 hypobaric rats that were killed the next day following 2 hours exposure to simulated high altitude. Two trials therefore, were necessary to complete each group i.e. 5 x 2 = 10/group.

All rats were killed at approximately the same time each morning (10:00 AM) therefore placement of the rats in the chambers to begin their trial was timed accordingly. After the exposure session, the rats were reweighed, anesthetized with ether and bled by cardiac puncture then organs removed and frozen for tissue analysis.

The measurements made to assess possible changes in free radical activity were as described in a previous study (details given in chap. II and III). The concentration of malonaldehyde (MDA) and lipofuscin were determined to assess the effect of the duration of the exposure on these indices of lipid peroxidation in tissues. As the concentration of cytochrome P-450 in liver has been shown to be increased by hypoxic conditions and to be decreased by the action of free radicals, this hemeprotein was compared.

Pulmonary edema often develops with altitude sickness in man and as this condition may reflect an increased permeability of the vascular membrane, the wet weight to dry weight ratio of this organ were compared. Hematocrit and methemoglobin indices which have been found to increase on exposure to hypoxia were also measured.

Statistical Analyses

A two-way analysis of variance with nonrepeated measures on all the indices measured was used to anal-

yze the data. Tukey's HSD(honest significant difference) was used in making comparisons between means. Although this was a series of independent studies the Pearson Product-Moment Correlation Coefficient, "r" was computed, using the mean values, to disclose possible relationships between some of the indices measured.

C. Results

The effect of the different durations of exposure to the hypobaric atmosphere on the tissues examined appeared quite variable. However, certain tendancies are evident. Application of ANOVA revealed that one or more of the exposure times had a significant effect on levels of each of MDA, lipofuscin, cytochrome P-450, as well as on lung water content and hematocrit. Only the level of methemoglobin appeared unaffected by the exposure in all instances.

The effects were not uniform on all tissues nor at all durations. Some differences evident with short exposure were not evident with longer exposure. For example, as may be seen in Table I, the increased concentration of MDA in the lung and kidney after 2 hours exposure suggests that lipid peroxidation was enhanced in these organs at this time. This increase in MDA was not evident in these organs of other rats that were exposed for longer times.

The decline in MDA concentration observed in the heart and liver after 2 hours followed by an increase suggests that peroxidation was initially depressed but becomes activated by some mechanism (free radicals ?) with longer exposure. Meanwhile its conversion to lipofuscin apparently continues as suggested by the increase in this in-

dex in the liver with continued exposure. The decrease in lipofuscin in the kidney suggests this organ may excrete this endproduct of lipid peroxidation.

As seen in the control values (Table I,II) withholding food and water for periods up to 24 hours did not affect lipid peroxidation in tissues uniformly. For example peroxidation in the lung (Table I,II) does not appear to be affected while in the kidneys of this group of rats, MDA concentration increased following 12 hours of food deprivation. In contrast, MDA concentration in the heart and liver was found to decline following 6 hours without food or water.

Table I. Malonaldehyde concentration in some tissues of rats following up to 24 hours exposure to hypobaric-hypoxia (6000m).

Exposure Time (Hr.)	Group	Pr. atm.	Malonaldehyde nmol.L^{-1} Lung	Kidney	Heart	Liver
0	Normals	1.0	184 (7)	352 (10)	302 (9)	595 (28)
2	Control	1.0	190 (4)	363 (11)	240 (21)	559 (17)
	Hypo-baric	0.5	231 a (7) b	490 a (8) b	161 a (17) b	470 a (15) b
6	Control	1.0	200 (4)	343 (14)	230 b (9)	312 b (17)
	Hypo-baric	0.5	188 (7)	402 (29)	381 a (10) b	476 a (34) b
12	Control	1.0	190 (17)	501 b (30)	311 (34)	433 b (31)
	Hypo-baric	0.5	199 (7)	454 b (21)	390 (33)	609 a (53)
24	Control	1.0	175 (6)	375 (18)	302 (33)	426 b (7)
	Hypo-baric	0.5	175 (4)	293 a (18) b	374 b (19)	463 a (10) b

Values = mean of 10 means, S.E.(in brackets)

Sign. diff. indicated: a vs control same column, same duration
$p<.05$ b vs normals

Table II. Lipofuscin fluorescence intensity in some tissues of rats following up to 24 hours exposure to hypobaric hypoxia (6000m).

Exposure Time(Hr.)	Group	Pr. atm.	Lipofuscin* Lung	Kidney	Heart	Liver
0	Normals	1.0	12 (.80)	78 (3.1)	43 (1.1)	71 (1.6)
2	Control	1.0	14 (.80)	76 (1.3)	42 (1.3)	79 (2.7)
	Hypo-baric	0.5	9 a (.40) b	74 (2.4)	52 a (1.2) b	67 a (1.5) b
6	Control	1.0	13 (.60)	65 b (1.3)	42 (1.0)	73 (3.5)
	Hypo-baric	0.5	14 (.66)	75 a (2.0)	44 (.9)	78 (1.8)
12	Control	1.0	14 (1.3)	70 (3.0)	45 (1.4)	90 b (1.9)
	Hypo-baric	0.5	15 (1.4)	66 b (2.1)	39 (1.4)	84 b (1.6)
24	Control	1.0	12 (.35)	61 b (1.1)	47 (1.6)	91 b (1.1)
	Hypo-baric	0.5	12 (.36)	62 b (1.5)	40 a (1.5)	88 b (1.3)

Values= mean of 10 means, S.E(in brackets)

* expressed relative to butadiene fluorescence(10^{-7}mol)

Sign. diff. indicated: a vs control same column, same duration
$p<.05$ b vs normals

As may be seen in Table III the duration of the exposure to hypobaric-hypoxia had a significant effect on the concentration of cytochrome P-450. Withholding food and water or exposure to simulated high altitude for periods up to 24 hours alterred (in most instances) the concentration of this hemeprotein. After 2 hours, total cytochrome P-450 concentration increased by approximately 47% in both groups of rats, however, after 12 hours of withholding food and water and/or exposure to hypobaric hypoxia, these stressors or other condition associated with the exposures appears to have increased destruction of this protein. Since the analysis of P-450 was done in random order the changes in levels cannot be due to systematic error. Further exposure (i.e. up to 24 hours) which resulted in an increase in P-450 in the liver of control rats suggests that processes in cells favoring synthesis of this protein were facilitated. In contrast to this apparent reversal of conditions in liver cells caused by withholding food and water (control rats), those exposed to simulated high altitude do not demonstrate this tendancy to P-450 synthesis at this time. Apparently, prolonging the exposure to hypobaric conditions suppressed and/or counteracted the effects that withholding food and water had on the concentration of cytochrome P-450. For example, withholding food and wa-

ter for 12 hours caused a 72% reduction in the total concentration of P-450(control vs normals) while only a 36% decrease in P-450 was found in the hypobaric rats during the same period of exposure.

Progressive weight loss, as may be seen in Table III, occurred in both groups of rats. The rats exposed to altitude, in all instances, lost progressively more weight than the control rats. In most instances the rats exposed to hypobaric-hypoxia lost more of their liver weight than the controls, perhaps due to dehydration.

Table III. Cytochrome P-450 in the liver of rats following up to 24 hours exposure to hypobaric-hypoxia (approx. 6000m).

Exposure Time(Hr.)	Group	Pr. atm.	Wt.loss (g)	Liver (g)	Cytochrome P-450 nmol*
0	Normals	1.0	-	18.1 (.85)	376
2	Control	1.0	8 (1.5)	16.3 (1.0)	549 b
	Hypo-baric	0.5	12.8 (1.6)	17.0 (.67)	557 b
6	Control	1.0	15.1 (1.7)	16.8 (1.2)	453 b
	Hypo-baric	0.5	16.5 (1.2)	13.6 a (.58) b	467 b
12	Control	1.0	17.6 (.77)	12.4 b (.70)	106 b
	Hypo-baric	0.5	25.2 a (1.6)	11.8 b (.35)	239 a,b
24	Control	1.0	32.1 (1.6)	13.2 b (.50)	256
	Hypo-baric	0.5	45.3 a (1.9)	11.5 b (.41)	204 b

* total P-450/liver

Values = mean of 10 rats, S.E.(in brackets)

Sign. diff. indicated: a vs control same column, same duration
p<.05 b vs normals

The increase in lung water content seen in Table IV as an increased wet weight to dry weight ratio was evident after 2 hours exposure to the hypobaric atmosphere. This change was no longer evident after longer exposures, in fact at 24 hours in the hypobaric atmosphere the water content of lung tissue was significantly [illegible] than that of controls. Withholding food and water [illegible] to 24 hours appears to have had little or no effect on the water content of the lung. Hematocrit showed a consistent increase with exposure, it being affected more by the "altitude" exposure than by withholding food and water. The concentration of methemoglobin appeared unaffected by the exposure.

Table IV. Lung water content of rats following up to 24 hours exposure to hypobaric-hypoxia (approx.6000m).

Exposure Time(Hr.)	Group	Pr. atm.	Wet/Dry ratio	Lung water(%)	Hct.	MetHb.* (Abs.)
0	Normals	1.0	3.92 (.12)	74 (.71)	45.9 (.66)	.39 (.01)
2	Control	1.0	4.24 (.12)	76 (.63)	46.6 (1.3)	.39 (.01)
	Hypo-baric	0.5	5.40 a (.27) b	82 a (.89) b	48.6 (.40)	.40 (.01)
6	Control	1.0	4.58 (.21)	78 (.70)	47.6 (.50)	.41 (.01)
	Hypo-baric	0.5	4.38 (.26)	77 (.86)	50 a (.70) b	.40 (.01)
12	Control	1.0	4.10 (.12)	76 (.65)	49.6 b (1.0)	.39 (.01)
	Hypo-baric	0.5	4.31 (.08)	77 (.75)	50.1 b (.60)	.39 (.01)
24	Control	1.0	4.00 (.17)	75 (.85)	48.9 b (.80)	.40 (.01)
	Hypo-baric	0.5	3.27 a (.10) b	70 a (.60) b	51.5 b (.70)	.39 (.01)

Values =mean of 10 means, S.E.(in brackets) for Hct. and MetHb., mean of 10 rats, S.E.(in brackets) for wet/dry and lung water

Sign. diff. indicated: a vs control same column , same duration
$p<.05$ b vs normals

* qualitative

Treatment of these data by ANOVA revealed that exposure of these rats to simulated high altitude increased peroxidation in the heart (Table I), induced synthesis of cytochrome P-450 in the liver and enhanced weight loss (Table III) and increased hematocrit (Table IV).

Withholding food and water for up to 24 hours significantly increased the concentration of MDA in some tissues (Table I, e.g. kidney-12 hours) and apparently enhanced its removal in others (Table I, e.g. liver-6 hours). Withholding food and water appears to have a significant effect on liver function (Table III, cytochrome P-450), however, this stress appears to have had little or no effect on the water content of the lung (Table IV).

D. Discussion

This series of studies on rats exposed to simulated high altitude for up to 24 hours provides some evidence in keeping with the suggestion that free radical-mediated reactions might be responsible for inducing changes in MDA, cytochrome P-450 and the lung water content. In most instances, the apparent decrease in these indices with longer exposures suggests that the conditions in cells causing the initial increases were reversed, terminated, or compensated for on continued exposure. This apparent reversal of conditions in some tissues of these rats, evident after a certain period of adjustment (acclimatization), could be considered to be analogous to the means by which amelioration of symptoms of altitude sickness occurs. When adequate rest or other precautions such as limiting exercise are taken, symptoms of altitude sickness typically become less severe. This suggests that the reactions within cells causing the symptoms to develop were terminated or compensated for on continued exposure, responses not unlike those found in these experiments. For example, the process which caused fluid accumulation in the lung observed at 2 hours is apparently reversed or compensated for with longer exposure thus potentially fatal pulmonary or cerebral edema did not develop in these rats.

The lung appears to be particularly sensitive to a reduction in atmospheric pressure. Increases in movement of fluid through membranes, such as the pulmonary vascular membrane, may result from either an increase in hydrostatic pressure difference across the membrane, an increase in membrane permeability or relative changes in colloid osmotic pressures between blood and interstitial fluid. An increase in pressure in the pulmonary arterial system has been observed in man and other mammals exposed to hypobaric conditions. Such an increase in pressure, along with possible changes in membrane properties, might have contributed to fluid accumulation evident in the lungs of these rats within 2 hours. Free radical-induced changes in properties of membranes which have been shown by other authors including Halliwell and Gutteridge(1984), Repine and Tate(1983),Deneke and Farburg(1980),Ohmori et al.,(1978) to increase membrane permeability may be one of the factors that contributed to the increase in lymph flow found by Levine et al.,(1988) to occur in sheep exposed to hypobaric conditions but not in sheep exposed to normobaric hypoxia. Since their studies also demonstrated that the increase in pulmonary pressure was the same during exposure to both types of hypoxia, some other factor must have caused the increase in lymph flow during exposure to hypobaric conditions. Levine and co-workers did

not assess the possibility of oxidative damage to tissues in their study. The present studies on rats provide evidence (MDA,lung water-2 hours,TableI,IV,respectively) to suggest that enhanced oxidation of pulmonary membranes at this time, resulting in a possible increase in permeability may have been a factor leading to accumulation of water in this tissue. The apparent decrease in the accumulation of water over the longer exposure suggests that the processes causing membrane damage were diminished or conditions reversed for water which may have accumulated was reabsorbed. The concomitant reduction in MDA(Table I) at this time, supports this suggestion.

The finding of an increased hematocrit (Table I) in rats after 24 hours exposure to altitude might reflect water loss including that due to hyperventilation or diuresis causing general dehydration of tissues including the lung. The reduction in MDA in the lung on continued exposure suggests less free radical damage to this tissue and thus a possible decrease in membrane permeability to osmotically active molecules. This apparent adjustment in free radical activity on continued exposure may have been a contributing factor.

The processes which determine the level of free radical activity within tissues are not clear. The variability in the concentration of MDA(Table I) assayed in var-

ious tissues of this study and in the concentration of cytochrome P-450 in the liver (Table IV) suggests that the immediate effects of withholding food and water or exposing rats to simulated high altitude on these indices are not the same(chap.III). Subsequent determinations of MDA in the lung,kidney,heart, and liver after 6, 12, or 24 hours exposure (Table I) suggests further the absence of a consistent response to these stresses and that the duration of the exposure evidently can affect free radical action in tissues. In some organs, particularly the liver (Table I,Table II, Table III-cytochrome P-450), exposure to hypobaric conditions appears to be suppressing or counteracting the effects that withholding food and water has on these indices. In others the longer exposure evidently enhanced oxidation in the heart (Table I), weight loss (Table III), and increased hematocrit (Table IV).

How the duration of the exposure to these different stresses can affect free radical action and thus the levels of certain indices in tissues in not evident from this study. However, one possibility is that glycogen stores in tissues are likely to become depleted with progressively longer periods without food or water thus affecting blood glucose concentration which in turn may have affected free radical action. This is suggested

by findings of Hearse et al.,(1976) and Rinette et al., (1984) that adequate glucose in the perfusing fluid can minimize free radical-mediated cell injury in tissues studied in vitro. That blood glucose levels and glycogen stores in tissues are depressed in rats by exposures of 24 hours or more to altitude or fasting has been demonstrated by some authors including Evans(1934), Cori(1932), Timaras et al.,(1958), Westerterp (1977), Ziegler(1967) and Conlee et al.,(1976). In the present study, differential rates of conversion of glycogen to glucose in different tissues during exposure to altitude or withholding food and water may have affected free radical activity and thus the levels of the indices measured, in particular MDA and cytochrome P-450. Some authors (Kappis and Sies,1981;Wolff et al.,1986;Reed and Pace, 1980) have suggested that without adequate substrates such as glucose or glycogen certain free radical scavenging mechanisms may be less effective. They have suggested that these substrates are essential to maintain glycolysis and the regeneration of GSH, NADH and NADPH, cofactors which are necessary for some free radical scavenging enzymes. Barber and Bernheim (1967) have shown that prior starvation can greatly enhance the sensitivity of the liver to the damaging effects of lipid peroxidation and suggested that the injury may have been caused by a decrease

in GSH thus enhancing the accumulation of free radicals. Perhaps, the decreased food intake associated with acute exposure to hypoxia found by Ettinger and Staddon (1982) and as found in this study enhances this effect. The present findings which demonstrate enhanced free radical activity in some tissues of rats after fasting are in line with the findings of Barber and Bernheim.

The trends in the concentration of cytochrome P-450 found with time in the liver of rats seen likely reflects the combined effects of withholding food and water and exposure to altitude on cell metabolism. Perhaps, variations in the concentrations of reducing equivalents in cells with time contributed to the changes seen in P-450. Wolff et al.,(1986) suggested that enhanced destruction of proteins often occurs when depletion of cellular reductants such as NADH, NADPH or GSH occurs. Shiraki and Guengerich (1984) concluded that the concentration of microsomal enzymes is primarily a function of changes in rates of protein synthesis as opposed to rates of degradation. The present findings suggest that the duration of the exposure may have affected mechanisms regulating the turnover of P-450. Sadano and Omura (1982) and Kumar et al.,(1980) found complete turnover of P-450 in rat liver to occur in 12 to 16 hours. The decrease in total P-450 found in the control rats (Table III) after 12

hours of withholding food and water followed by an increase in the concentration of P-450 in the controls at 24 hours would support their findings.

The lack of formation of methemoglobin under these conditions suggests that the antioxidant mechanisms of the red cell, which includes various cofactors, were adequate to prevent the formation of methemoglobin or superoxide.

In conclusion, nonuniform changes in MDA, lipofuscin, and cytochrome P-450 suggests that free radical activity may be enhanced in some but not all tissues of rats by withholding food and water and/or by exposure to hypobaric hypoxia. Furthermore, these findings suggest that the magnitude and nature of these reactions, including possible changes in membrane permeability, may differ with the duration of the exposure to these conditions and other possible stresses.

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Chapter V

Conclusions and Recommendations

This series of studies was undertaken to determine if acute exposure to hypoxic atmospheres might enhance free radical activity in tissues of rats thus possibly damaging membranes. They provide some evidence to support this hypothesis. Increased levels of MDA found in the lungs and kidneys following 2 hours exposure to hypobaric-hypoxia suggests that free radical-induced lipid peroxidation was likely enhanced in these organs. At the same time lung water also increased significantly suggesting that the vascular membrane in the lung was rendered more permeable by these conditions. The findings, in part, are similar to those of Varskeviciene et al.,(1984) who found in rats, a marked increase in MDA concentration only in lung tissue following 2 hours exposure to hypobaric-hypoxia(an atmospheric pressure of 200 mmHg). They did not measure lung water. The decrease found in the total concentration of cytochrome P-450 in the liver following up to 24 hours exposure to hypobaric-hypoxia is in line with a possible increase in free radical activity in this organ. These mechanisms i.e., enhanced lipid peroxidation and protein degradation, which may be related to the action of free radicals, appear to be reversible or compensated for on continued exposure, similar to the initial symptoms of acute mountain sickness in man. Similar reactions in tissues of man, if they occur during acute exposure to high altitude, might

underly the development of various forms of edema as well as other symptoms of mountain sickness including intravascular hemolysis, hematuria or proteinuria. All these symptoms may reflect damage to membranes leading to increased permeability of small proteins and water.

The effects of exercise at altitude on the variables studied is of interest as this stressor is recognized to aggravate the symptoms of acute mountain sickness. Barcroft (1914) hypothesized that mountain sickness can be triggered by exercise. Since his initial observations many others have corroborated his findings. The findings of the present studies, that lipid peroxidation is enhanced by exposure to altitude, along with previous findings of other authors (Corbucci et al.,1894;Davies et al.,1982;Dillard et al.1978 and Lovlin et al.,1987) that it is enhanced by exercise alone suggests that the exacerbation of mountain sickness by exercise reflects the combined effect of the two variables on lipid peroxidation. Whether or not this reflects a combined effect of the two variables on lipid peroxidation remains to be determined.

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