# Semi-Annual Status Report

NASA Grant NGR 06-002-075

31 October 1972



Colorado State University
Fort Collins, Colorado

TABLE OF CONTENTS

## ABSTRACT

During the past six months, the Laboratory of Aerospace Biology has completed two and initiated several new studies toward describing the mechanism of inert gas influence on metabolism. Dr. Christopher Schatte completed his work with Dr. Peter Bennett at the Royal Naval Physiological Laboratory in England which dealt with the acute metabolic response to frank sensory narcosis. They found that elevated pressures of nitrogen and helium accelerate metabolic rate and shift the metabolism towards greater usage of fatty acids at the expense of carbohydrate. Conversely, nitrous oxide narcosis depresses metabolic rate and appears to favor the utilization of carbohydrate for oxidation.

The hamster has been considered a possible subject for a flight experiment based on its relatively low amount of excrement and small size. In collaboration with Dr. X.J. Musacchia, hamsters were exposed to a mixture of 80% argon: 20% oxygen at one atmosphere and 70% $O_2$: 30% $N_2O$ at 0.33 atmospheres to ascertain whether or not these animals responded in a manner similar to rats. A reduced oxygen consumption, lower food intake and a loss of weight were observed in argon over a three day period. A similar though less pronounced trend was seen in the hypobaric $N_2O$ environment relative to air controls. Both the nature and degree of the metabolic response was the same as that which we have recorded in rats under similar conditions. The hamster, therefore, will be a suitable model for a flight experiment involving metabolic depression using inert gaseous environments.

The emphasis of our search for the mechanism of action of inert gases has shifted primarily to the cellular and subcellular level. Dr. Schatte has initiated a study to confirm reports that nitrous oxide might produce a histotoxic hypoxia. He is measuring the oxygen consumption and production of "excess" lactate in humans during rest and exercise while breathing a mixture containing $N_2O$ or air. By calculating the differences in oxygen debt in these two gases, a comparison of oxygen utilization within the cell can be had such that a histotoxic hypoxia would be reflected in a decreased oxygen uptake and increased lactate production.

Dr. Schatte has also begun a study of narcotic susceptability as a function of membrane fatty acid composition. Mice are being reared under conditions of temperature and diet such that the mitochondrial and plasma membranes of their cells will contain a high proportion of either polyunsaturated or saturated fatty acids. The animals will be tested in argon, a known metabolic depressant, and elevated pressures of air to determine both the metabolic response and the susceptibility to a narcotic situation as a function brain fatty acid composition. If narcotic gases exert their influence at lipid sites in the membrane, we expect to see a differential response of the experimental animals to the test environments relative to control.

David Clarkson has begun his dissertation research involving a multi-faceted program at the whole body, cellular, and sub-cellular levels. The whole body experiments are designed to complete our investigation of the thermal vs. non-thermal effects of diluent gases. Rats will be placed in a chamber such that the head is exposed to mixtures of He, Ne, Ar, $N_2$, and $N_2O$ while the remainder of the body is in air at the thermal neutral temperature. The oxygen consumption, $CO_2$ production and catabolism of radiosubstrates will serve to compare the metabolic effects of each mixture at a constant thermal requirement for the body.

Similar measurements will be performed on disaggregated liver and brain cells in vitro using the above gases plus krypton and xenon. By eliminating thermal, hormonal, and neural factors, the response of individual cells to the gases will serve to further challenge the presence of a direct metabolic effect. Intact cells will also be used for the measurement of intracellular oxygen tensions in the presence of different gases. Pyrenebutyric acid (PBA) will be used to estimate oxygen concentration based on fluorescence. If diluent gases do have an ability to alter oxygen flux across the cell membrane, the intracellular oxygen concentration should be affected.

Upon confirmation of the metabolic effects at the whole body and cellular levels, analysis of sub-molecular changes will be investigated. Rats will be exposed for 24 hours to mixtures of He, Ne, $N_2$, Ar and $N_2O$ in the large metabolic chamber system. While still exposed to the test environment, the animals will be decapitated, the brain and liver removed, and the mitochondria and cell membranes prepared from these tissues. The mitochondria will be analyzed for Kreb's cycle intermediates using gas chromatography and respiratory control ratios determined in the presence of the test gas. Cell membrane fractions will be exposed to the test environments in the

presence of ANS and changes in membrane configuration measured by fluorescence spectroscopy.

Mr. Clarkson will thus have examined most of the possible sites of diluent gas action and will be able to relate his cellular and sub-cellular findings with those which have accumulated in whole animals over the past few years.

In addition to the above investigations, we are using hamsters to systematically test the metabolic rate parameter using $^{14}CO_2$ expired from injected acetate-1-$^{14}C$ which we have developed over the years. The results will be submitted as a new method to the Journal of Applied Physiology.

Finally, this report contains a proposal for collaborative research among the Regulatory Biology group of NASA Life Sciences. The proposed research involves the use of hypoxia to combat cardiovascular deterioration in the weightless state and is designed to provide a flight experiment protocol as the ultimate phase of the study.

## INTRODUCTION

During the period covered by this report, Dr. William Martz completed his post doctorate here and assumed a position as assistant professor of biochemistry at Sangamon State University, Springfield, Illinois. While here, Dr. Martz broadened the scope of our work by investigating the effects of diluent gases on brain synaptosomes. The initial findings, reported in the 30 April 1972 Status Report, indicate that oxygen consumption of synaptosomes is reduced by argon, in accord with our whole animal findings. Acetylcholinesterase activity also appears to be affected but the nature of the change is not clear. We will continue his work with the intention of characterizing diluent gas effects at both cholinergic and adrenergic synapses.

Dr. Christopher Schatte completed his post-doctoral tenure in England and returned to assume responsibility for day-by-day operations in the Laboratory of Aerospace Biology (LAB). While in England, Dr. Schatte performed collaborative research with Dr. Peter Bennett at the Royal Naval Physiological Laboratory which was concerned with the metabolic effects of frank narcosis. A preprint of the manuscript resulting from his work is included in this report. On the return from England, Dr. Schatte attended the Fifth Symposium on Underwater Physiology in Freeport, Grand Bahamas. This meeting has traditionally been attended by the world's foremost authorities in the fields of closed environments and inert gas narcosis. In addition to the information gathered at this meeting, arrangements were made with Dr. Thomas Akers of the University of North Dakota for a joint research effort between LAB and his group. Dr. Akers is currently studying the influence of diluent gases on acetylcholine release in vitro; his knowledge will aid us in the pursuit of the synaptosome work.

The scrubber train of the metabolic chamber system has been modified by replacing the sulphuric acid scrubbers with a water trap consisting of a micropore sediment bowl and two silica gel canisters. Ammonia is trapped by an additional canister filled with activated charcoal. Not only has trapping efficiency appeared to have improved but we have also eliminated a major source of system malfunction and breakdown. These modifications, in conjunction with the mixing of boric acid

with Pel-E-Cel in the drop trays, have provided improved control over chamber humidity and ammonia.

In the 30 April 1969 Status Report, we described a mathematical method for quantifying expired $^{14}CO_2$ from injected radiosubstrates. The constant use of this procedure since then has confirmed its validity and uniqueness. We have decided to initiate a formal study of the method preparative to its publication in the Journal of Applied Physiology. This study is just beginning and we expect to have a manuscript for submission prior to the next Status Report.

In addition to the above, research projects either performed or initiated during the past six months are described in subsequent sections of this report. They include: (a) measuring the metabolic response of hamsters to narcotic gases in collaboration with Dr. X.J. Musacchia of the University of Missouri; (b) experiments to determine the effect of fatty acid composition of cells on susceptibility to inert gas influence; (c) beginning of tests with humans to ascertain the interaction between a narcotic gas, $N_2O$, and oxygen at the cellular level; (d) experiments with tracheotomized rats to conclude our work comparing the thermal versus non-thermal effects of diluent gases; and (e) molecular level studies investigating diluent gas-induced changes in tricarboxylic acid cycle intermediates and intracellular oxygen concentration.

Finally, we have included a proposal for cooperative work within NASA Life Sciences' Group in which we would study a possible means of reducing cardiovascular deterioration in the weightless state.

## METABOLIC RESPONSE OF HAMSTERS TO ARGON AND NITROUS OXIDE

The probability of using hamsters in a space flight experiment has motivated us to compare their metabolic behavior with that of the rat exposed to various diluent gases. At the urging of Dr. X.J. Musacchia of the University of Missouri, we exposed a group of his specially-bred hamsters to argon at one atmosphere and nitrous oxide ($N_2O$) at 0.33 atmosphere to ascertain whether or not the metabolic depression we have characterized in rats would occur.

A group of eight hamsters was exposed for three days to each of the following environments: air, Ar-$O_2$, air, $N_2O$-$O_2$ at 5psia, air. Ambient temperature was maintained at 22 $\pm$ 1°C. Food (Lab chow) and water were available ad libitum. Food consumption and weight change were measured as the difference between pre- and post-exposure values for each environment. Oxygen consumption was calculated on the basis of oxygen added to maintain a set point on the Beckman F-3 analyzer. Readings were taken at four hour intervals between 0800 and 2400 hours each day.

Table 1 shows the results of oxygen consumption, food intake and weight change, three parameters which we have found to predictably change with the diluent gas. The mean values indicate that the general metabolic depression previously observed in rats exposed to argon and $N_2O$ also occurs in hamsters. Argon exposure significantly reduced all three indices with respect to both pre- and post-exposure controls. The lower oxygen and food consumption and loss of weight are consistent with our data from rats. Nitrous oxide at a partial pressure of less than 100 mmHg did not have as profound an effect but nevertheless appeared to reduce metabolism relative to pre-exposure control. The fact that the post-exposure control period did not show a return to normal is probably a systematic effect of the experimental protocol rather than a gas-dependent one.

We have found that recovery in air following exposure to an exotic gas mixture is slower than the changes attendant upon initiating the exposure. If complete recovery is not allowed to take place prior to testing another diluent gas, the metabolic

responses in the latter case will not be artificially smaller than they should be. Complete recovery normally takes 5-7 days; accordingly, our exposure periods did not allow a full return to normal values. The progressive decline in the air control values for oxygen consumption and weight change are indicative of inadequate recovery during the course of the experiment.

Despite the abbreviated nature of the data, it is apparent that hamsters respond to diluent gases in the same manner as do rats. The use of diluent gases in a flight experiment involving hamsters is therefore feasible and the results representative of the mammalian population in general.

Table 1. Mean values for $\dot{V}O_2$ weight change, and food consumption during exposure of eight hamsters to argon at one atmosphere, nitrous oxide at 0.33 atmospheres, and air at one atmosphere.

| | Air | Argon | Air | $N_2O$ | Air |
|---|---|---|---|---|---|
| $\dot{V}O_2$, L/kg/hr | 1.70 | 1.40 | 1.65 | 1.58 | 1.52 |
| Weight change, g/kg/day | 27 | -18 | 12 | 0 | 0 |
| Food consumption, g/kg/day | 65 | 49 | 54 | 50 | 59 |

## MEMBRANE FATTY ACIDS AND SUSCEPTABILITY TO NARCOTIC GAS INFLUENCE

Of the theories formulated to explain the mechanism by which inert gases exert biological effects, perhaps the most plausible postulates interaction of the gas molecules with the lipoid component of cellular membranes (1). This interaction may cause the membrane to expand (2), reduce its surface tension (3), or undergo conformational changes (4). Whichever the case, membrane function is affected probably due to a change in the properties of the sol and gel state.

Interchange between these two phases is largely a function of the fatty acid composition of the membrane, particularly that of phospholipids (5). It may be significant that narcosis, which presumably alters the conditions necessary for phase change, can be alleviated by the use of cationic detergents, acetylsalicylic acid, and Vitamin E; each of these compounds is known to act on the non-polar phase of the membrane (6).

If the narcotic state does alter the ability of a membrane to undergo a phase change, it is possible that altering the membrane's composition might serve to allow normal phase changes in the presence of narcotic gases. Such a situation does occur in cold adaptation. It has been shown that the degree of unsaturation in membranes increases with cold acclimation (7). The membrane thus stays "fluid" at temperatures which would normally cause it to be "stiff". That this allows phase changes of membranes at low temperatures has recently been demonstrated in vitro (8).

By feeding a highly unsaturated fat diet to animals at a low ambient temperature, their cold tolerance has been shown to improve relative to controls and animals fed a highly saturated fat diet at a warm ambient temperature (9). It can be inferred from this work that membrane composition was altered in terms of the degree of saturation of the fatty acids.

Perhaps artificially changing the fatty acid composition of brain and skeletal muscle might influence the susceptibility to narcotic gases. We intend to raise mice from birth on a diet and at an ambient temperature designed to make their membranes more or less saturated with respect to controls. The pups will be transferred after birth into one of three experimental treatments:

(1) 45% safflower oil diet, 5°C ambient temperature

(2) 45% beef tallow, 35°C

(3) standard laboratory chow, 20°C

Two dozen mice will be raised under each of these conditions for three months such that most growth will have occurred under the desired circumstances. At the end of the treatment period, the animals will be tested for narcotic susceptability using both metabolic depression and anesthetic endpoint as parameters. The degree of reduction of $\dot{V}O_2$ upon exposure to argon as compared with air at one atmosphere will be used as the metabolic measurement. The pressure in atmospheres of air required for loss of consciousness will be used as the anesthetic endpoint. This particular test will be done in conjunction with Dr. Thomas Akers at the University of North Dakota.

After testing is complete, the mice will be sacrificed and samples of brain, liver, and skeletal muscle analyzed for fatty acid composition. Initially, total hydrolyzable fatty acids will be determined; if significant differences exist, analysis will be run for composition of specific phospholipids and triglyceride fractions.

If the hypothesis that control of membrane fatty acid composition will change narcotic susceptibility is correct, we anticipate that mice with more unsaturated membranes will show lesser metabolic depression in argon and a higher air pressure required for anesthesia relative to controls; conversely, the highly saturated fatty acid mice should prove more susceptible to the two tests.

The results of this study, if positive, will provide insight into the site and mechanism of inert gas action on membranes and could aid in developing treatment to either reduce or enhance their narcotic effects.

## NARCOSIS-INDUCED HISTOTOXIC HYPOXIA

As part of our continuing effort to control metabolic rate using inert gases, we have examined nitrous oxide ($N_2O$), a moderately potent narcotic gas with relatively little toxic effect. Several reports indicate that it causes hypoxia during its use in anesthesia (10). Such an action might represent one means by which it exerts its effect. It is of particular interest to us because we have previously noted the apparent ability of diluent gases to modify the severity of an imposed hypoxia at the cellular level (11). The consistency of these results with those reported for $N_2O$ could represent a universal characteristic of diluent gases which might assist in explaining their mechanism of action.

We are commencing a study to describe the influence of $N_2O$ on oxygen utilization within the cell as determined by the oxygen debt incurred during exercise. That $N_2O$ has such an effect can be inferred from the work of Bradley (12) who found that humans breathing 30% $N_2O$ had a lower consumption than in air both at rest and during two levels of exercise. Thus, $N_2O$ appears to either modify the body's need for oxygen or, more likely, prevented normal oxygen utilization. If the latter concept is true, the cells must have incurred a greater oxygen "debt" in $N_2O$ than in air.

We are repeating Bradley's experiments with emphasis on parameters which reflect oxygen debt. By measuring oxygen consumption, $CO_2$ production, blood lactate and pyruvate, we will have an indirect assessment of the degree of anaerobic metabolism in the presence of $N_2O$ relative to air. If any differences in the onset and recovery from oxygen debt do occur, it will be proof that $N_2O$ does alter cellular oxygen uptake.

The testing will use humans as subjects breathing either air or a 30% $N_2O$: 50% $N_2$: 20% mixture of oxygen, which has a narcotic potency roughly equivalent to 7-10 ATA compressed air. Each subject will be tested in a resting and an exercise mode.

For the resting phase experiments, steady state oxygen consumption and $CO_2$ production will be measured while breathing air, $N_2O$, then air. Each gas will be respired for 30 minutes, the last 10 of which will be used for data collection, to insure good equilibration with the body. Other parameters will include heart and

respiration rate, minute ventilation and rectal temperature.

Exercise experiments will consist of two trials, one in air and one in $N_2O$, on separate days. The subjects will breath a mixture for 20 minutes after which a blood sample will be drawn from the antecubital fossa. All sampling and medical supervision for the experiment will be done by a licensed anesthesiologest in the Department of Physiology and Biophysics.

After adequate equilibration with the test gas, the subject will exercise for 5 minutes on a bicycle ergometer and rest for the following 20 minutes. Gas analysis and physiological measurements will be taken throughout the equilibration, exercise and recovery periods. Blood samples will be drawn at 5, 10, 15, and 20 minutes after the beginning of exercise.

Blood samples will be analyzed for lactate and pyruvate and the "excess" lactate calculated according toHuckabee(13) as a measure of anaerobiosis and oxygen debt. Patterns of ventilation, respiratory gas exchange and heart rate will be compared at rest and during exercise for the two gases. Additionally, the rate of "repayment" of the oxygen debt during the recovery period will be assessed. If $N_2O$ does depress oxygen consumption, we expect to observe a larger oxygen debt with a longer recovery in $N_2O$ than in air.

Once the presence or absence of $N_2O$-oxygen interaction is established, we can undertake long-term exposures of animals with the knowledge that reductions in oxygen consumption, which we have previously observed, caused by $N_2O$ are not due solely to its sedative effect. Further, any potential use of $N_2O$ on a prolonged basis will be inherently safer if such a phenomenon is acknowledged and taken into consideration.

## A BIOCHEMICAL STUDY OF INERT GAS NARCOSIS

The object of the project outlined in the following is a better definition of the metabolic response of a mammalian animal to the inert gases, i.e., those in the noble gas series: helium, neon, argon, kryptron, and xenon as well as nitrogen, as it relates to the narcotic potency ascribed to these gases. From this it may be possible to describe a general mechanism for the induction of narcosis. Accordingly, this project is divided into three phases: I. The metabolic response of whole animals as a consequence of their exposure to the inert gases; II. The metabolic response of intact cells on exposure to the inert gases; and III. The metabolic and macromolecular response of certain subcellular components exposed to the inert gases.

I. The investigation of whole animal metabolism on exposure to the inert gases is intended to strengthen and extend the observations made in this laboratory in the past four years that metabolic rate is significantly influenced by the type of inert diluent gas, i.e., that gas when mixed with oxygen represents the major component in the breathing atmosphere. Additionally, this phase of the investigation will differentiate between the heat transfer characteristics (influencing the rate of heat conduction away from the body surface) of these gases, which in themselves may well influence metabolic rate, and a more direct metabolic affect. This is especially critical since the principal fodder of contemporary criticism is that the observed changes in metabolic rate correlate not only with the various physical properties of the inert gases, e.g., molecular or atomic weight and lipid solubility, but with their thermal conductivities (Table 1) as well. Thus, arguments concerning the direct metabolic effects of these gases (principally the lighter ones) are lost for failure to adequately account for this one physical parameter.

There are three, and perhaps only three, routes of approach to controlling the metabolic influence of this parameter. In the first method the thermal conductivities of the various gas mixtures are adjusted to approximate that of normal air by changing

Table 1. Calculated thermal conductivity (k) of inert gas-oxygen mixtures in the ratio of 4:1 (15).

| GAS | k x $10^6$ (cal/$^oC$-$cm^2$-sec) |
|---|---|
| $He:O_2$ | 228.77 |
| $Ne:O_2$ | 96.71 |
| $N_2:O_2$ | 56.88 |
| $Ar:O_2$ | 44.22 |

the partial pressure of the diluent gas while maintaining a constant $P_{O_2}$. This requires a change in the total environmental pressure which introduces another variable- that of the effect of pressure on metabolic rate. This latter effect has not been well characterized and consequently provides a point for argument against the direct metabolic effect of inert diluent gases when this approach is used.

The second method for controlling the differences in the thermal conductivities of these gases is to perform the metabolic measurements within the thermal neutral range, i.e., the range of environmental temperature within which the experimental animal maintains a constant metabolic rate and compensates for slight environmental temperature fluctuations by physically controlling body heat balance, as determined for animals exposed to each of the gases at a fixed diluent to oxygen ratio. This method, although avoiding the ambiguity of the effects of pressure differences, does not provide good resolution between thermal effects and direct alteration of metabolic activity by the diluent gas under study.

A third method which is currently under investigation is the isolation of the experimental animal's respiratory system from the rest of the environment. Such a maneuver allows the animal to be placed in one type of gaseous environment while breathing a gas mixture of an entirely different type. Thus, air can be used as an external reference gas, so to speak, and direct metabolic affect of the selected diluents evaluated in a rather uncomplicated way.

Initially, it seemed that chronic tracheal intubation would be the most desirable

way of respiratory isolation. Several trials were performed on laboratory rats (Holtzman) in which a piece of stainless steel tubing (hypodermic needle stock) 4-5 mm in length was inserted into the trachea immediately posterior to the larnyx through a ventral incision in the tracheal wall. Respiratory failure attributed to tracheal mucus secretion rapidly occurred in all cases. Neither substitution of polyethylene catheter tubing for the stainless tubing nor pretreatment of the rats with atropine sulfate (0.4 mg/kg, I.P.) effected significant improvement of this technique.

Consequently, a different approach was attempted. The trachea was exposed, severed immediately posterior of the larnyx, brought to the ventral surface just anterior of the clavical and sutured in place. Excess skin was trimmed away to prevent the formation of a pocket around the tracheal orifice. These rats were similarly pretreated with atropine sulfate; additionally, this group received local injections of dexamethasone (2 mg) and epinephrine (0.1 cc, 1:1000) postoperatively. These preparations failed within twelve hours as a result of blood clot formation over the tracheal orifice. The cause of this formation was attributed to excessive activity of the animal and perhaps abuse of the incision by digging at it with the hind feet. This can most likely be prevented by fitting a collar around the neck of the animals which prevents access to the incision but does not restrict airflow.

Generally, tracheal cannulation or exposure do not appear to be particularly promising approaches primarily from the standpoint that the profuse mucus secretion induced by abrasion of the interior tracheal surface will have to be suppressed by chronic doses of anti-inflamatory steriods and/or epinephrine. Either drug could bias the desired metabolic measurements. Additionally, the lengthy preparation time necessary for the number of animals required makes this approach to respiratory system isolation somewhat unappealing.

Perhaps a better method, which is currently being tested, is to fit the experimental animal with a hood which seals around the neck and through which breathing gases can be flowed. The only difficulty encountered with this procedure is obtaining an adequate seal between the hood and the animal - a problem which, it is felt, can be easily surmounted.

The following is the protocol for this series of experiments: on experiment days a male rat (400-450 gm) is selected from the colony, fitted with a gas hood and thermo-

couples (deep body, tail and dorsal skin) placed in a hardware cloth restraining cage (to prevent the dislocation of the thermocouples or removal of the hood), and placed in a small thermostated chamber. The hood is connected to a supply of air or a synthetic gas mixture, i.e., an inert gas and oxygen in a ratio of 4:1. The chamber is sealed and constantly purged with a small volume of the reference gas. Connections between the rat and instrumentation are made through bulkhead connectors in the chamber wall. The chamber temperature is maintained at 28°C ±.5°C which is within the air thermal neutral range of the rat (16). To guard against cross contamination of the hood and chamber gas the hood and chamber effluent gas streams are periodically sampled and analyzed by gas chromatography.

For assessing the metabolic activity of the animal under these conditions the effluent hood gas is passed through a paramagnetic oxygen analyzer (calibrated to correct for $CO_2$), a infrared spectrophotometer for $CO_2$ analysis, and into a caustic liquid $CO_2$ trap. The trap serves as a backup system for the real-time system as well as providing a means of trapping $^{14}CO_2$ when labeled substrates are injected into the experimental animal. The concentration of oxygen and carbon dioxide is recorded continuously on strip chart recorders for subsequent analysis.

The animals body temperatures as well as chamber temperature are recorded manually at 15-minute intervals. A method for the continuous recording of these data is being developed.

Under the current experimental design each animal serves as its own control and all exposures are at ambient pressure (632 mmHg). Body temperatures, oxygen consumption and carbon dioxide production are measured for two hours with the animal on air then, for two hours on a synthetic gas mixture. The sequence is periodically reversed to correct for temporal bias. Further, thirty minutes are allowed for equilibration after switching the animal to a gas of different composition before any data are recorded. In addition, the composition of the external reference gas is changed while the animal is breathing air, thus permitting an assessment of metabolic activity attributable to the thermal conductivity of the environment. Exposure is limited to the following inert gases: helium, neon, nitrogen, argon and nitrous oxide. The use of krypton or xenon in this series is not contemplated because of their exorbitant cost.

In conjunction with oxygen and total carbon dioxide data the rate of production of 14-$CO_2$ from acetate-1-$^{14}C$ and glucose-1-$^{14}C$ is being used as an indicator of gross metabolic activity. Half of the experimental animals are injected (I.P.) with sodium acetate-1-$^{14}C$ and half with glucose-1-$^{14}C$ at a dose rate of 25-50 $\mu$Ci/kg; the animals are injected just after equilibration with the test gas or air. Labeled carbon dioxide production rate is determined by counting an aliquot of samples taken periodically from the carbon dioxide trap following injection of the radio-labeled substrate.

II. a. Past studies of the effect of inert gases on the metabolic activity of various tissue preparations have yielded data which are difficult to interpret on the basis of whole animal experiments. Some data suggest that heavier gases, e.g., argon and xenon, may accelerate metabolic rate while other data suggest no effect whatever (17,18,19). There are several possible explanations for such discrepancies. For example, a metabolic response may be dependent upon a highly integrated system - the whole animal - or the altered metabolic activity may be a summation of events occurring at the cellular level, i.e., a function of mass rather than the degree of integration. In the latter case the methods typically used to assess cellular metabolic activity (manometry) may not be adequately sensitive. There is also the distinct possibility that in some instances the tissue selected for study may be relatively insensitive to the presence of inert gas, e.g., many studies have been conducted with liver tissue. Possibly some other tissue such as brain or myocardial tissue would be a better choice. Further, previous metabolic studies have been almost exclusively performed with either tissue slices or homogenates rather than washed whole-cell preparation. The latter would seem to offer the advantage of a better defined system than those typically used.

To better define the role of cellular metabolism in observed whole animal response to the inert gases, the following experiments will be performed. Rats will be exposed in groups of eight to a gas mixture containing oxygen mixed with one of the inert gases (as in section I, plus krypton and xenon) for 24 hours. (Control animals exposed to air will be treated similarily.) For the exposure, these animals will be housed in a sealed chamber in which the gas composition and temperature can be controlled. During the course of the exposure oxygen consumption and carbon

dioxide production will be monitored as an indication of metabolic activity. At the end of the 24th hour the rats will be decapitated and the brain and liver removed, weighed and rinsed in cold buffer solution. (The decapitation step and all subsequent steps will be performed in the same type of gaseous environment to which the rats were exposed and all solutions will be equilibrated with this test gas before use.) Subsequently, the tissues will be minced with scissors and transferred to a second cold buffered solution containing 3 mM tetraphenyl boron for disagregation of cells after the method of Rappaport (20). Disagregation will be assisted periodically by passing the tissue suspension through a series of pipetts of progressively smaller bore. On completion of this step (one to two hours) the suspension will be passed through cheese cloth to remove chunks of tissue which have not completely disagregated and the filtrate centrifuged at 200 xg for 5 minutes to harest the cells. The resulting pellets will be washed three times to remove as much debris as possible. The final preparations will be resuspended in a buffered solution (10 volumes) and examined microscopically for gross cellular damage. Those preparations exhibiting significant damage will be either subjected to further washing or discarded. Cell counts will be performed on each of the surviving preparations (using a hemacytometer) to correct subsequent data for differences in cell numbers. Each sample will be transferred to a polarographic apparatus (Yellow Springs Instrument Company) for measurement of oxygen consumption at 37°C. Carbon dioxide production will be quantified by infrared spectrophotometry after Neal and Jones (21) or alternatively by the use of caustic-wetted filter paper. The metabolic activity of these cell suspensions will be further characterized by use of sodium acetate-1-$^{14}C$ and glucose-1-$^{14}C$ providing an additional means of comparison between cell and whole-animal preparations. The amount of $^{14}CO_2$ produced will be quantified by collecting the $CO_2$ on caustic treated filter paper. The bicarbonate formed will eluted from the filter by extensive washing with additional caustic solution and precipitate will be collected on pre-weighed cellulose ester filters (24 mm diameter), dried, reweighed and counted by liquid scintillation spectrometry.

b. It has been suggested (22) that altered metabolic rate observed in whole animals may be the consequence of hinderance of oxygen difusion across the plasma membrane which effects a progressive intracellular hypoxia in going from the lighter inert gases, e.g.,

helium, to the heavier ones, e.g., argon. This is a difficult hypothesis to test, since there are no direct methods available to measure intracellular oxygen concentration which would not in some way damage the membrane, e.g., micropoloragraphic electrodes. Recently, however, a method has been suggested for measuring intracellular oxygen concentration remotely and which apparently leaves the plasma membrane intact and the cell metabolically competent (23,24). This is accomplished by measuring the oxygen quenching of fluorescence of pyrenebutyric acid (PBA). The fluorescence quenching of this fluorophore is linear over the oxygen concentration range of 0-700 mmHg. Additionally, PBA is readily accumulated by cells, thus greatly reducing the background fluorescence attributable to the suspension medium.

The most direct means of evaluating this hypothesis is to measure the time required to attain a steady state concentration of oxygen (as determined by the rate of change of fluorescence intensity) within a cell following the introduction of this gas into an anaerobic suspension of cells. To make things less complicated it would be desirable to perform this measurement on cells whose aerobic oxidative metabolism is blocked, since the rate of utilization of oxygen may affect its apparent rate of uptake. Although aerobic oxidation can be effectively blocked with such compounds as cyanide or amytal (25), it is not known whether the uptake of PBA by cells is an active process requiring aerobic metabolism or a passive process which requires only the maintainance of a concentration gradient by taking PBA out of the intracellular solution. In the former instance aerobic metabolism would very likely be required to maintain the gradient.

This can be effectively tested by exposing normal oxygen deprived cells (isolated as above) to PBA and determining the rate of uptake by spectrophotometrically measuring the extracellular concentration (after first removing the cells) at intervals following initial exposure. These data when compared with those on normal aerobic cells will provide a basis for the oxygen requirement for PBA uptake. Assuming a passive process the effect of the chosen metabolic inhibitor on PBA diffusion should be evaluated in groups of inhibited anaerobic cells and normal anaerobic cells, since the inhibitor might affect the diffusion process.

Assuming a straight forward situation where the accumulation of PBA is an entirely passive process and an aerobic state is not required for the maintenance of a concentration

gradient, normal cells isolated from rat liver and brain (by methods described previously) will be treated with a respiratory inhibitor and PBA. Subsequently, the suspension will be deoxygenated by equilibrating it with one of the inert gases and purging continued until all the oxygen has been removed. An aliquot of the suspension will be transferred anaerobically to a seal cuvette, placed in a spectrofluorometer and background fluorescence measured (excitation at 342 nm and emission at 370 mn). A small volume of oxygen saturated solution will then be injected into the cuvette and the rate of change of fluorescence intensity recorded either oscillographically or by means of a stripchart recorder. This will be done with each of the inert gases as in the preceeding section. Data will be compared with air exposed cells.

If the aerobic state is found to be an absolute requirement for PBA uptake and the maintenance of a concentration gradient, the following procedure will be adhered to. Cells in a sealed cuvette will be exposed to PBA in an oxygenated medium also containing an inert gas. When the oxygen tension of the suspension approaches the point at which the cells become anaerobic, as determined by a slowing of the rate of increase in fluorescence intensity, but before PBA begins to diffuse back into the medium, the suspension will be rapidly mixed with an oxygen-saturated solution. Oxygen diffusion rate will then be measured as before.

If this hypothesis proves to be correct, one would expect to see an attenuation in the rate of oxygen diffusion on going from helium to xenon or nitrous oxide. A negative result does not necessarily mean the hypothesis is incorrect, since the procedure as outlined above assumes the plasma membrane to be the principal target of these gases and does not account for oxygen permeability changes which could occur in the mitochondrial membranes. To this end it would necessary to investigate the permeability of mitochondria to PBA and proceed in a manner similar to that outlined above.

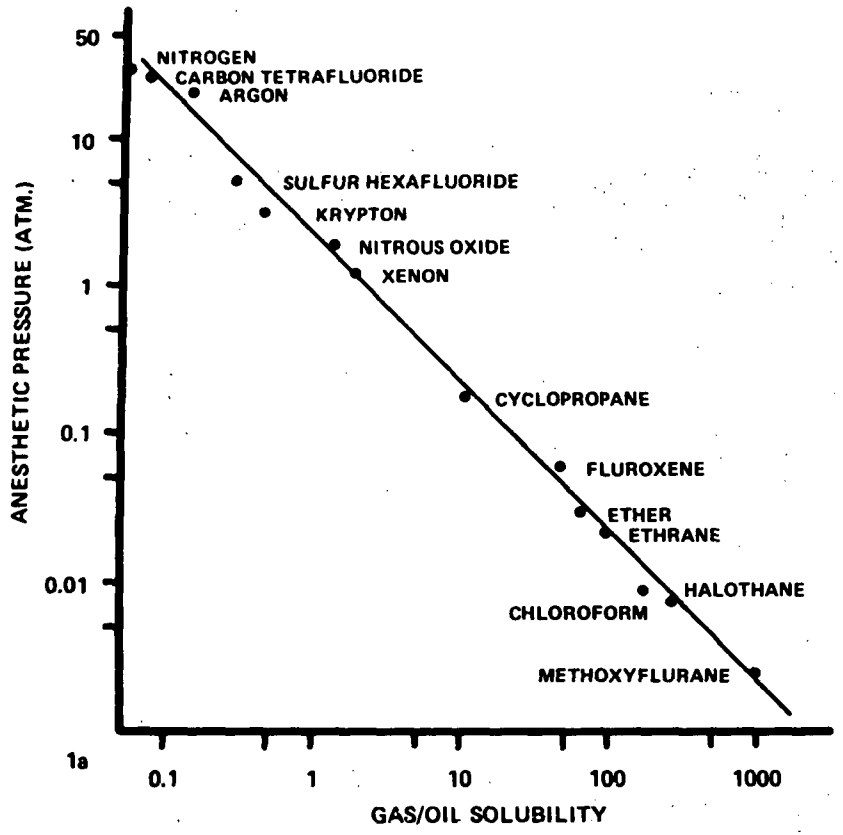
III. a. The classic theory on the mechanism of narcosis is based on the pertubation of biomembranes by the narcotizing agent. The narcotizing effect is attributed to the solution of the agent in the membrane which subsequently alters the activity or function, e.g., a change in permeability, of this structure. The mangitude of the effect is related to amount of the agent solubilized in the membrane. Thus, the more soluble

the agent is in membrane material, specifically lipid, the greater its narcotizing potency. This relationship is demonstrated in Figure (1a).

A more contemporary theory for the induction of narcosis is based on the stabilization of water by the narcotizing agent and the subsequent formation or stabilization of microcrystals or "icebergs" (26-27). Iceberg or microcrystal formation is believed to occur on the membrane surface and are stabilized by van der Waals forces not only by the disolved agent (a gas) but by membrane components, e.g., protein side chains. The ultimate effect of augmented water structuring is believed to be the stabilization of the membrane with attendant alteration in permeability and possibly other activities. The principal argument against this theory is that many gaseous narcotizing agents are effective at partial pressures below or above those usually required for the formation of the gas hydrate. For some agents the discrepancy is very great (Figure 1b). Because of the limited knowledge concerning the conditions for gas hydrate formation, as it applies to the living organism, this theory is not widely accepted.

Regardless of which theory proves to be the more correct it would be interesting to investigate the physical behavior of biomembranes when exposed to gaseous narcotizing agents. So far as is known, this has not been done presumably because methods for doing this have only recently been introduced. Perhaps the most sensitive in vivo or in vitro method is the use of a fluorophore, e.g., 8-anilino-1-naphthalene sulfornic acid (ANS).

ANS and similar naphthalene derivatives have the rather interesting property that their fluorescent intensity is proportional to the dielectric constant of the solubilizing medium (29). Thus, the fluorescence intensity of ANS increases several hundredfold when associated with hydrophobic material, e.g., lipid, relative to an aqueous environment. Although ANS manifests a high affinity for protein, when used with membrane preparations it is found to be almost exclusively in the lipid fraction (30,31). The association of ANS with the membrane fraction appears to be a solubilization process rather than physical binding (32). Recently, ANS has been used to study cation interaction with sarcoplasmic reticulum (30), conformational changes in $Na^+, K^+$-dependent AT Pase (33), and apparent changes in membrane conformation concurrent with energy coupling in intact mitochondria (29) and submitochondrial



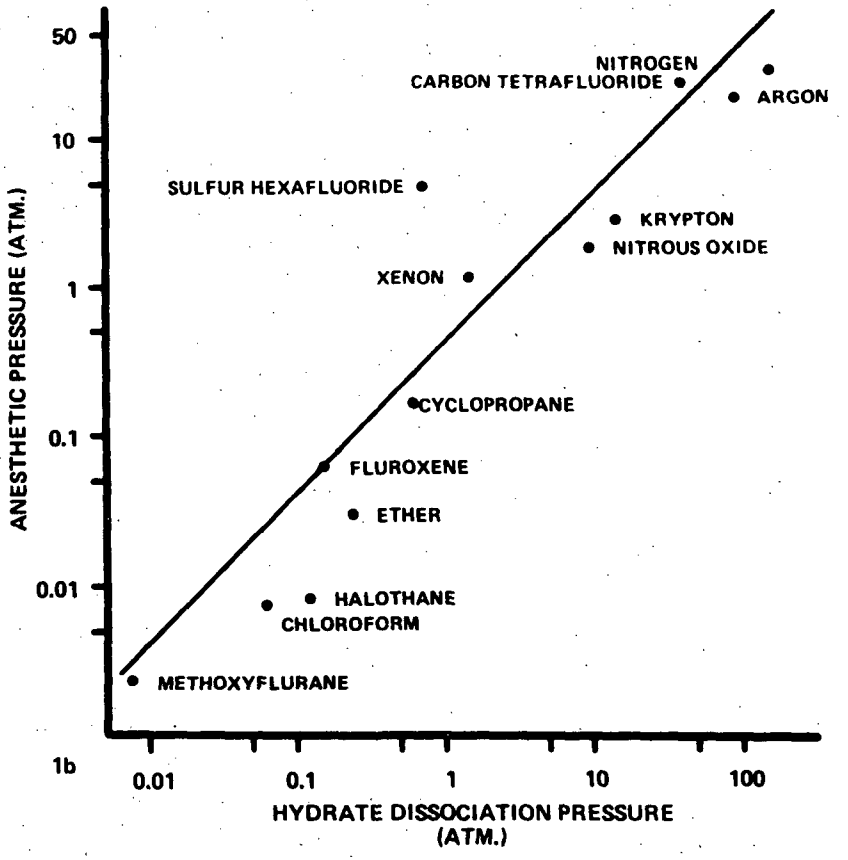




FIGURE 1. The graphs indicate the correlation between the partial pressure in atmospheres (atm.) of the anesthetic or inert gas required to supress physical response to painful stimuli and (a) the gas/oil solubility ratio of the gas or (b) the pressure in atmospheres at which gas hydrate (clathrate) crystals breakdown. These date are interpreted as indicating that the inert gas or ansethetic activity is primarily in the lipid phase, i. e. membrane, as opposed to the inhancement of water structuring at the membrane surface or within the cell. Figure re-drawn from reference (28); data for nitrogen, argon, and krypton from reference (27,39).

particles (34). If membrane conformation changes are attendant to narcosis this method appears to have the requisite specificity and sensitivity to reveal such changes.

Liver tissue will be taken from rats previously exposed only to air and prepared as described in Section II a. Aliquots of the resuspended pellet containing a known number of cells will be lysed and the plasma membrane fraction purified as described by Hinton (35). The plasma membrane fractions will be transferred along with 10 um of ANS to cuvettes fitted with a gas-tight plug. The cuvettes will be incubated in the cell holder of the spectrofluorometer for a brief time and initial fluorescence intensity and polarization of fluorescence will be recorded (excitation at 370 nm and emission at 450 mn). The cuvette will then be purged with chosen diluent mixture (as in Section II) until the residual nitrogen has been washed out (as determined by gas chromatography) and the measurements repeated. A change of fluorescent intensity or polarization, representing changes in hydrophobicity and microviscosity respectively, will be taken as a gas-related physical change in the membrane. The release or addition binding of ANS, which will affect intensity measurements will be monitored by pelleting the membranes and determining the relative concentration of ANS in the supernatant of control and experimental preparations.

This experiment will be repeated with intact cells. This type of preparation may prove to be unsatisfactory as a result of interference by ANS bound to other cell constituents which could mask a subtle, but otherwise measurable change.

III. b. Previous studies of the effect of inert gas on the metabolic rate of mitochondria did not yield significant results (18, 19). However, scrutiny of the methods used by these investigators revealed that in one preparation the mitochondria were subjected to a severe hypotonic shock before use; the other investigator used succinate as the sole substrate. Further, apparently neither investigator established that the mitochondria were competent, e.g., tightly coupled. Cohen (36,37) has recently demonstrated that both state III and respiratory control values of mitochondria are markedly depressed by physiological concentrations of frank anesthetics, e.g., halothane. By use of different substrates, Cohen concluded that the terminal NADH dehydrogenase of the electron transport system was the principal target of the anesthetics. Why this enzyme should be more sensitive to the presence of an anesthetic than any of the other enzymes of the inner mitochondrial membrane is not readily apparent. Similar

behavior of mitochondrial respiration has been reported for diethyl ether and chloroform (38).

If, as Figure (1a) suggests, the inert gases are a part of the continuum formed by the conventional anesthetics, e.g., nitrous oxide, ether, halothane, etc., it should be possible to demonstrate a progressive decline of mitochondrial respiration corresponding to the progressive increase in the oil solubility of the inert gases in going from helium to xenon (Table 2).

Table 2. Oil solubilities (Bunsen coefficient) of the inert gases at 22°C (39).*

| GAS | SOLUBILITY |
|---|---|
| He | 0.015 |
| $N_2$ | 0.052 |
| Ar | 0.15 |
| Kr | 0.44 |
| Xe | 1.9 |

* Data not available for neon.

To test this hypothesis, mitochondria from the liver and brain of rats will be prepared according to the methods of Johnson and Lardy (40) and Basford (41), respectively. The mitochondria will be used as soon as possible after preparation, but stored as a pellet on ice until use. Just prior to use the mitochondria will be taken up in the buffered salt solution (42) with the following modifications: N-2-hydroxyethylpeperazine-N'-2-ethanesulfonic acid (43), 10 mM at pH7.4 will be substituted for the phosphate buffer system and 1 mM of monopotassium phosphate will be added as the phosphate donor. The amount of protein in suspension will be quantified by the 280/260 ratio method of Warburg (44). An aliquot of suspension will be transferred to small flask into which a modified fast-response polarographic electrode (45, 46) is subsequently inserted. The flask will be warmed to 37°C, the oxygen baseline established and approximately 100 uM of adenosine diphosphate will be transferred to the medium. The respiratory control ratio calculated from these data will serve as an indication of the competency of the mitochondrial preparation; typically, such values should lie between 4 and 10 (40).

Subsequent aliquots of the mitochondrial suspension will be assayed for their ability to utilize the following substrates: glutamate, succinate, and β-hydroxybutyrate. Once air baseline values have been established the flasks will be flushed with one of the diluent gases (mixed with oxygen) and the respiratory control ratio and substrate utilization measurements repeated.

Although the reported data (36,37) do support the conclusion that the respiratory activity of mitochondria is altered in the presence of anesthetics, the data do not seem sufficient to warrent the conclusion that NADH dehydrogenase in specifically affected, e.g., P/O ratios were not determined. Since P/O ratios cannot be reliably calculated from ADP/O ratios (47), this phosphate production (48) and oxygen consumption will be determined on preparations similar to those described above, but including hexokinase-glucose system as a phosphate trap. Additionally, to evaluate the direct effect of inert gas on the activity of the Krebs cycle the intermediates of this cycle will be quantified. The intermediates from mitochondria in state III. respiration and exposed to one of the inert gases will be prepared and analyzed by gas chromatography (49,50).

## HYPOXIA-INDUCED PROTECTION AGAINST CARDIOVASCULAR DETERIORATION IN THE WEIGHTLESS STATE

The Regulatory Biology Group, of which LAB is a part, will meet at Colorado State University in November to formulate a series of experiments culminating, it is hoped, with a proposal for a flight experiment. Such a program must deal with a problem of interest to the NASA mission, must entail an experimental protocol which can be performed only during a space shot, and the results should lead to a practical application of the techniques to manned space flight.

As a suggested program which the group as a whole, and LAB in particular, might undertake, we propose to address ourselves to the question of cardiovascular deterioration attendant with exposure to weightless ness. This problem is of current interest to the NASA mission, it involves experimentation with the weightless state which can only be achieved on a practical basis during space flight, and the solution to the problem will probably be applied to missions in the future.

If the solution is to be applied to man in the near future, experimental protocols must incorporate a treatment suitable for man. Hypothermia has been demonstrated to attenuate biological changes under adverse conditions but producing hypothermia in men as a standard operating condition is not a feasible procedure within the near future. The use of drugs is likewise not a preferable treatment on a chronic basis. Further, any remedy should represent a constant and continuous treatment since weightlessness is constant stimulus while out of planetary gravitational fields. Thus, intermittent exercise has not proved entirely successful in offsetting the loss of vascular tonus in space.

We propose to use chronic hypoxia as a remedial treatment to obviate the effects of weightless ness. It is well documented that hypoxia causes cardiovascular hypertrophy such that animals acclimatized to low oxygen tensions have a "stronger" circulatory system than normoxic ones. By reducing the inspired oxygen tension during flight, there would be a constant but passive stimulus of an astronaut's cardiovascular system, which if of sufficient degree, might be made to offset the hypotonicity resulting from loss of gravitational stimulus.

The proposed program would entail three phases of experimentation terminating with the flight experiment.

PHASE I.

The initial experiments would be a preliminary study to determine the feasibility of the approach. A group of hamsters, an ideal animal for a space experiment, would be acclimatized for one month to an hypoxic mixture of nitrogen and oxygen while control animals were maintained in air. The hypoxia would be sufficient to cause some tachycardia and hyperventilation during normal activity but not so as to appreciably change metabolic rate or growth; the probable level would be about 12% oxygen in nitrogen at one atmosphere, equivalent to approximately 15,000 feet altitude.

Once physiological acclimatization has progressed as indicated by red cell, hemoglobin and plasma protein levels, the two groups of animals will be tested for circulatory "strength" each week for the next two months. The test will involve the ability of the animal to withstand systemic vasodilation while in the vertical position, a measure of the system's ability to supply the brain with blood against the force of gravity.

Each animal will be placed individually in a plexiglas cylinder with wirecloth on the inside surface to allow a foothold. The cylinder will be positioned such that the hamster will be inclined on its back about $30^{o}$ from vertical. He will be able to move only up or down on the wirecloth. The bottom of the cylinder will be lined with wirecloth and connected via a power supply to the vertical wirecloth. If the hamster touches bottom, a shock will be received from below. The animals will be trained to stay off the bottom of the cylinder in this manner.

The cylinder will be sealed with the hamster clinging in the vertical position, flushed with 100% oxygen, and the animal allowed to settle down for five minutes. At this point, an amount of amyl nitrite sufficient to produce syncope and "blackout" will be injected into the cylinder and the time until loss of consciousness, as evidenced by the animal sliding to the bottom, measured. Upon touching bottom, the amyl nitrite will be purged with oxygen, a shock stimulus given, and the time to reattainment of equilibrium and climbing off the bottom recorded.

This procedure will allow a crude measure of cardiovascular resistance to gravitational stress. The ability of the system to supply the brain with adequate blood flow (a function of cardiac rate, contractility, and vasomotor tone) in the face of low central venous pressure will serve to approximate the relative fitness of the two groups. If hypoxia does improve cardiovascular tone, we would expect to see a greater time to blackout and quicker recovery as hypoxic adaptation progresses.

A group of hamsters which have been conditioned to severe exercise will also be tested as a reference point for cardiovascular tone. Since exercise improves circulatory system tonus, largely due to hypoxic drive during exercise, the validity of the testing procedure can be determined. It is expected that the animals made chronically hypoxic will respond intermediate to the exercised hamsters and air controls.

Two other testing techniques which might work as an alternative are the use of heat stress or lower body negative pressure to produce severe vasodilation.

PHASE II.

If the preliminary testing indicates that this is a feasible approach, a more detailed series of experiments would be undertaken. A large group of hamsters would be maintained at altitude on Pike's Peak such that permanent acclimatization could occur in animals born and raised at altitude. These animals would be used for detailed physiological studies including the measurement of red blood cell indices, arterial and venous pressures, cardiac output and tissue enzyme levels, all of which classically change during adaptation to hypoxia (14). Experiments would be performed testing the altitude-acclimated hamsters against sea-level controls in the centrifuge at elevated g loads. If they tolerate high g levels sufficiently well, the ultimate experiment in this phase would be to breed and raise hamsters at 2g in air and a low oxygen environment. Upon reduction to one g, the responses of the two groups would serve to approximate the situation of concern, namely, reduction of gravity towards zero after acclimatization to one g.

PHASE III.

True zero gravity would be tested in a space shot. Four animals born and raised at altitude would be tested against a similar number which were raised at sea-level. The actual flight portion of the experiment could be simple or relatively complex, as desired. The simplest experiment would involve exposing them to weightlessness in their accustomed environments and testing them upon return. During flight, any parameters which would not influence cardiovascular tonus could be measured.

If the flight allowed more sophistication, the testing could be done in space so as to show response vs. duration of exposure relationship. This would require a centrifuge positioned such that the animals could be herded into the compartment and rotated at a force of 1g head to tail. An appropriate parameter, perhaps the amyl nitrite test, would serve to determine physiological response to the granitational stress. Time of blackout and cardiac rate at that point could be monitored via implanted EEG and EKG leads connected to a transmitter on the animal's back. If the space shot were manned, testing in space could be performed with relative ease and assurance.

## COMMENT

If hypoxia does present a means of attenuating the cardiovascular decrement associated with weightlessness, its use as a space capsule atmosphere would be preferable, from several standpoints, to hypothermia, gas narcosis or pharmacological agents. Hypoxia is a natural phenomenon to which man and animals adapt easily and which allows, within the proposed limits, a normal mental and physical state. At the hypoxic levels proposed, metabolic rate would not be appreciably affected although growth rate might not be as great as normal. If desired, hypothermia, narcosis or some other altered state could be used in conjunction with hypoxia.

Perhaps the most important point is that hypoxia is readily reversible by the addition of oxygen. Thus, if an astronaut were preparing for heavy work or a crucial maneuver requiring maximum mental activity, he would simply breath pure oxygen

for the duration. In such a situation, he would be in a better physiological state than the unacclimatized person. With more red cells and greater cardiovascular tonus, his oxygen delivery system would be much more efficient at any workload. He would have better blood flow due to increased vascularity induced by hypoxia. Tissue oxygen utilization would be more efficient due to increased enzyme activity caused by hypoxic stimulation. And, in situations requiring activity during an hypoxic state, i.e., heavy work or an emergency low oxygen circumstance, his stamina, endurance, and resistance to hypoxic symptoms would be much greater than normal.

We wish to propose this study as a collaborative project among different laboratories within the Regulatory Biology group. The cardiovascular testing, experimentation using a centrifuge and the metabolic measurements might best be done by individual laboratories with particular competence in each of these areas. Additionally, such cooperative work would better serve the interests of both the group and the NASA Life Sciences program by pooling both the experience and financial assets of the group towards a single but milti-faceted goal – a meaningful space flight experiment designed to better prepare for future manned habitation of outer space.

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# APPENDIX

The following paper entitled, "Acute metabolic and physiologic response of goats to narcosis", by C.L. Schatte and P.B. Bennett has been submitted to the editors of Aerospace Medicine for publication. The referencing and pagination are separate from the remainder of the Status Report.

# ACUTE METABOLIC AND PHYSIOLOGIC RESPONSE OF GOATS TO NARCOSIS

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## ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Mr. J. Towse, Mr. A. Dossett, Mr. M. English, Mr. J. Florio and Mrs. B. Andrews for their technical and diving assistance with the experiments. Further gratitude is extended to the staff of the hyperbaric chamber facility at RNPL under the direction of Mr. J. Eaton. We are particularly grateful to Mr. S. Gray, R.N.H. Haslar, for his advice and assistance with the biochemical analyses.

Christopher Schatte wishes to acknowledge his sponsorship at RNPL by Dr. John Patrick Jordan under the auspices of NASA grant NGR 06-002-075.

## ABSTRACT

The metabolic response of goats was assessed during a one hour exposure to 97% $N_2$: 3% $O_2$, 97% He: 3% $O_2$, or air at sevenatmospheres absolute and to 75% $N_2O$: 25% $O_2$ at sea level. Physiologic and metabolic parameters measured included heart rate, EEG, visual evoked response, EKG, body temperatures, oxygen consumption, $CO_2$ production, the uptake and oxidation to $CO_2$ of infused glucose-UL-$^{14}C$, and blood levels of glucose, pyruvate, lactate, and free fatty acids. Hyperbaric nitrogen caused an increase in metabolic rate and a general decrease in blood constituent levels which was interpreted as reflecting a shift toward fatty acid metabolism at the expense of carbohydrate. A similar but more pronounced pattern was observed with hyperbaric helium. Breathing $N_2O$ at sea level resulted in a substantially depressed metabolic rate and a perceived shift toward greater carbohydrate catabolism. These metabolic alterations are probably not detrimental during short exposures but could influence physical well-being over a prolonged period.

# INTRODUCTION

It is well established that exposure to elevated partial pressures of nitrogen and certain noble gases depresses CNS function in man and higher vertebrates (1). Perhaps less well known is the fact that these same gases at an ambient pressure of one atmosphere absolute (ATA) cause a change in metabolic rate and apparently can alter the relative activity of certain metabolic pathways ( 8 ).

It has been suggested that narcosis is caused by an association of a narcotic gas with the lipoidal component of cellular membranes such that the membranes undergo a physical change, i.e., expansion ( 9 ), reduction in surface tension ( 6 ). If such a configurational change does occur, it is reasonable to suppose that metabolic enzymes, which are part of the membrane, might be detrimentally affected by narcosis. If the condition were sufficiently prolonged, the resulting effects and any possible adaptation would become increasingly germane to the well-being of the subject.

With the realization of prolonged exposure to artificial environments for habitation in both inner and outer space becoming increasingly probable, it is mandatory that the metabolic consequences of exposure be fully assessed both in the normobaric and hyperbaric situation. Toward such an end, this study was undertaken to describe metabolic changes attendant with the narcotic state, with particular reference to initial alterations upon induction of narcosis.

## METHODS

Eight adult, female goats weighing 40-45 kg were trained to stand in a wooden restrainer and breath through a face mask at a pressure of 7 ATA. The animals were exposed individually to the test environments and were not used as subjects more than once every seven days. Subjects were starved 12 hours prior to an experiment to minimize variations in baseline metabolic parameters. Polyethylene cannula were inserted into both jugular veins either one day or immediately prior to the experiment.

All experiments began between 0900-1000 hours. A rectal thermistor was inserted, a skin thermistor taped posterior to the right scapula, and EEG and EKG electrodes positioned. At zero time, a priming dose of 20-30 $\mu$ Ci of glucose-UL-$^{14}C$ (2-4 mCi/mmole) was administered through the right jugular cannula and followed by a constant infusion of radioglucose at a rate of .02-.03 $\mu$ Ci in 0.5 ml saline per minute. The infusion lasted for three hours, the first of which was necessary to allow mixing throughout the body (11). As determined by plasma glucose specific activity, equilibration occurred during the second and third hours of the infusion and it was during these latter two hours that the pre-exposure control and experimental periods took place. Total injectate was either 60 or 90 $\mu$ Ci of glucose-UL-$^{14}C$ in 100 ml saline.

At the beginning of the pre-exposure control period, the animal with attendant was compressed to 1.15 ATA (5 FSW) in order to facilitate collection of expired gas from the chamber. All compressions were made with air. During these pre-exposure control periods, the goats breathed either air from the chamber or 80% He: 20% $O_2$ from a Douglas Bag. At ten minute intervals throughout the one hour control segment, EEG, frequency analysis (2), EKG, an evoked response (VER) using strobe lamps as a visual stimulus, and temperatures were recorded. Expired $^{14}CO_2$ was trapped in hyamine hydroxide after Shigekoto and Wagner (10) every ten minutes at the same time a blood sample was drawn from the left jugular cannula. Expired gas was collected continuously in Douglas bags which were sampled at 20 minute intervals for gas chromatographic analysis.

Upon completion of the pre-exposure control period, the subject plus attendant was compressed in two minutes to 7 ATA. The experimental protocol was repeated with the animal breathing either air from the chamber, 97% $N_2$: 3% $O_2$ or 97% He: 3% from a Douglas bag. After the one hour period at pressure, decompression with oxygen stops was carried out with no incidence of bends using the 1968 Royal Naval Decompression Tables.

Two experiments each were performed in which the hour at pressure was replaced by an hour at 1.15 ATA breathing either air or 75% $N_2O$: 25% $O_2$. In these cases, the noise of compression was simulated at the beginning of the final hour of the experiment.

Each experiment entailed the withdrawl of 144 ml blood which represented less than 5% of the total blood volume. The simultaneous infusion of 100 ml saline and at least one week interval between experiments using the same subject reduced any complications due to blood loss.

Physiological data collected included heart rate, frequency analysis of the EEG with particular reference to alpha and theta wave activity, an averaged evoked response as described by Bennett ( 2), and skin and rectal temperatures. Expired gas analysis was performed using dual Perkin-Elmer gas chromatographs equipped with mol sieve 5A and Poropak "Q" columns. Helium was the carrier gas for experiments using $N_2$ or $N_2O$ while argon was used as carrier for samples containing helium. The oxygen and carbon dioxide content of each 20 minute sampling period was used to calculate oxygen consumption and $CO_2$ production. Expired $^{14}CO_2$ specific activity was determined by direct counting of the hyamine hydroxide coctail containing one millimole of $CO_2$ ( 10).

Blood samples were immediately divided into tubes containing NaF-heparin or 1N perchloric acid. The tubes were slowly decompressed one hour after termination of the experiment; hemolysis was rarely observed. Plasma glucose was measured enzymatically in an Auto-Analyzer. Plasma lactate, pyruvate and free fatty acids were determined using the respective Boehringer kits. Plasma glucose specific activity was measured by liquid scintillation counting of the pentaacetate derivature ( 7 ).

Statistical analysis was carried out where possible using student's "t" test; differences with chance probabilities of less than 5% were considered significant.

## RESULTS

The results are presented, and should be interpreted, with the following considerations in mind. Despite training, the goats were obviously stressed by the experimental situations. Most animals remained still during the testing period but showed signs indicative of a stress response including pupil dilation, occasional hyperventilation, elevated body temperatures, increased heart rate and higher than normal blood constituent levels. Referring to the air control values in Table 1, it can be seen that almost every parameter measured decreased during the second hour when compression would have normally occurred. This is suggestive that the animals were settling down after having been excited; such a settling process probably did not occur in experiments involving compression. The typical stress response involves neural and hormonal-mediated metabolic changes which characteristically affect almost every parameter with which we are concerned. Since the degree of stress was not uniform in every case nor can its contribution be assessed relative to the tested variables, our data may be a function of and differentially affected by a stress response. Although we have quantified on a relative basis, the observed effects are probably valid only on a qualitative one.

Air or $N_2$-$O_2$ at 7 ATA

The percentage change in the various parameters for nine experiments in which the goats breathed air at the surface and air or 97% $N_2$: 3% $O_2$ at 7 ATA are shown in Table 1. There were no apparent differences between air or $N_2$-$O_2$ at depth so that the data were pooled. Both gases produced substantial narcosis based on a reduction of EEG spikes during VER and suppression of alpha and theta wave activity. Both $O_2$ consumption and $CO_2$ production were elevated and the relatively greater increase in oxygen uptake significantly depressed RQ. Based on respiratory gas exchange, metabolic rate was increased in nitrogen at pressure.

With regard to the blood parameters, it is pertinent that these values are weighted toward the metabolic activity of the brain. Although left jugular blood contains blood constituents which have circulated from other parts of the body, parameters such as glucose, lactate and pyruvate which are measured in jugular veinous blood are primarily a function of brain metabolism. Glucose-related parameters in particular are representative of brain function because in the non-lactating ruminant, the brain accounts for a greater portion of total body glucose utilization than in non-ruminants.

Apart from an increase in plasma glucose concentration, the blood constituents measured tended to decrease with depression of lactate being significant. The depression of FFA was not as great as that in the surface controls such that nitrogen at 7 ATA actually increased plasma FFA slightly relative to surface control in the absence of compression. Plasma glucose was elevated at pressure but was probably more a function of excitement rather than of narcosis.

Changes in the rate of radioglucose uptake from the blood and appearance of $^{14}CO_2$ were interpreted as reflecting changes in glucose utilization. The infused glucose equilibrated with the body during the second and third hours of the infusion. At equilibrium, the rate of glucose entry into the blood was approximately equal to the rate of its removal by cells from the blood. Thus, the specific activity of plasma glucose should have been relatively constant during this equilibrium if these conditions were met. An effect of the experimental treatments on either glucose uptake or its conversion to $CO_2$ would cause a change in the specific activity of plasma glucose or $^{14}CO_2$, respectively.

By measuring the slope (m) of a line determined by least squares regression using the specific activities determined in the pre-exposure control and experimental hours, the relative rate of change in specific activity of both glucose and $CO_2$ can be determined. In Figure 1 , it can be seen that a greater decrease in the slope for glucose specific activity occurred in nitrogen at pressure than in the surface control experiments suggesting that glucose was taken up at a greater rate under pressure. Conversely, the change in slope of $^{14}CO_2$ specific activity was about the same in both environments. It can be inferred from these data that glucose was taken up at a greater rate at pressure but was not oxidized to $CO_2$ any faster. Therefore, some shunting of glucose away from the oxidative pathways must have occurred. One possible alternative is conversion to fatty acids for metabolism, a postulate which is not inconsistent with a lowered RQ and increased fatty acid utilization.

Rectal and skin temperatures significantly decreased at pressure but this appeared to be a trend of the experimental protocol rather than a gas effect since similar temperature changes occurred in the surface control experiments.

He-$O_2$ at 7 ATA

Exposure to 97% He: 3% $O_2$ at 7 ATA was intended to assess the effects of pressure in the absence of narcosis relative to breathing 80% He: 20% oxygen on the surface. The VER and EEG parameters indicate that CNS depression was not evident in helium when compared to nitrogen at pressure. We were unable to obtain acceptable oxygen consumption data in helium. But the lower values at the surface relative to surface controls and hyperbaric nitrogen, in conjunction with an increase in $CO_2$ production of the same order as that in nitrogen, makes reasonable the assumption that any increase in oxygen consumption while breathing helium at depth was probably no greater than that in nitrogen.

Juxta-opposed to the situation in nitrogen, there was a general increase in blood constituent levels while breathing hyperbaric helium. The greater increase in plasma lactate relative to pyruvate caused an increased ratio. From this, it can be presumed that a relatively lesser amount of pyruvate was shunted to the acetyl CoA pool or TCA cycle at pressure.

An increase in plasma FFA further suggests that, if the relative contribution of pyruvate to acetyl CoA and the TCA cycle was lower, fatty acid catabolism by the TCA cycle may have increased. That an increase in non-carbohydrate catabolism may have occurred is also suggested by the radioactivity parameters. The slopes m indicate that the rate of glucose uptake and conversion to $CO_2$ was reduced at pressure. It should be noted, however, that some difficulty was encountered in obtaining the $^{14}CO_2$ samples at pressure. These results are based on only a single successful experiment and thus may be less representative of the actual situation.

It is interesting that heart rate did not significantly decrease at depth since diving bradycardia is thought to be a pressure phenomenon. It would seem that, in this specific instance, the absence of narcosis may have altered the chronotropic response to pressure.

$N_2O$-$O_2$ at one atmosphere

A mixture of 75% $N_2O$: 25% $O_2$ was tested at sea level to produce a more profound narcosis than that observed in nitrogen at 7 ATA and thereby magnify the metabolic changes caused by narcosis. The goats were able to stand and respond to audio stimuli but were clearly more narcotic based on visual observation. However, the EEG manifestations of the narcosis were not as apparent as with hyperbaric nitrogen. The VER spikes were less depressed relative to pre-exposure control than in hyperbaric helium. While characteristic alpha wave suppression occurred, theta wave activity was actually enhanced by $N_2O$.

In contrast to hyperbaric narcosis, oxygen consumption was significantly depressed while $CO_2$ production was substantially increased. The rather large increase in expired $CO_2$ may have been partially artifactual. Although care was taken to separate the $N_2O$ and $CO_2$ peaks on the gas chromatograph, overlapping probably occurred. The RQ values of up to 2.06 suggest an unphysiologically elevated $CO_2$ expiration. Alternatively, there may have been an increase in $CO_2$ excretion from the rumen, a factor which can differentially influence $CO_2$ production measurements and RQ calculations for ruminants. It is probable that whole body $CO_2$ production increased but not to the extent indicated by the data.

Plasma lactate decreased, similar to nitrogen, but pyruvate significantly increased and thereby caused a significant drop in lactate: pyruvate ratio. This suggests an increased production and shunting of pyruvate away from lactate formation and toward the TCA cycle. Perhaps in response to this, plasma FFA decreased sharply and RQ rose with the shift away from fatty acid catabolism.

Glucose uptake did not change appreciably from normal but a greater proportion of it was oxidized to $CO_2$ during $N_2O$-narcosis, an implication consistent with the presumed shift toward greater carbohydrate utilization.

It should be noted too that breathing $N_2O$ produced no significant change in rectal temperature and an increase in skin temperature, findings which are in contrast to the patterns seen in all other experiments.

## DISCUSSION

Our results indicate that induction of a narcotic state does cause metabolic alterations but the nature of the changes are dependent upon the narcotic gas and the pressure at which its breathed. Exposure to hyperbaric helium, an essentially non-narcotic situation, produces different metabolic effects which are presumed due to pressure alone.

As measured by oxygen consumption, nitrogen at 7 ATA produces an increase in metabolic rate while $N_2O$ breathed at one atmosphere depresses it. Hyperbaric helium probably accelerated metabolic rate to the same degree as did nitrogen at the same pressure. It is therefore possible that changes in respiratory gas exchange in hyperbaric nitrogen were a function of pressure rather than the diluent gas; but the depressed $O_2$ consumption in $N_2O$ was almost certainly a gas-related effect.

Apart from changes in metabolic rate, it can be inferred that shifts in the relative activity of different metabolic pathways also took place. For a relatively crude approximation, the rate of glucose uptake and increases in plasma levels of pyruvate and lactate can be used to assess glycolytic activity. Oxidation via the TCA cycle is reflected in the rate of appearance of $CO_2$ and oxygen consumption. Differences in the plasma lactate: pyruvate ratio can be used to estimate the relative amounts of pyruvate being converted to lactate versus transfer to other pathways, primarily fatty acid synthesis and the TCA cycle.

Based on these generalized concepts, exposure to nitrogen at 7 ATA was considered to cause an increased glucose uptake without a commensurate increase in its appearance as $CO_2$. The lowered lactate and pyruvate levels suggested that glycolysis was not increased and that the extra glucose was shunted out of the glycolytic pathway before reaching the TCA cycle. Its ultimate destination is not clear but it may have been incorporated into lipid metabolic pathways. Although plasma FFA was depressed relative to pre-exposure controls, the magnitude of reduction was not as great as in the surface controls and therefore could be viewed as actually having increased above normal. Fatty acids may have been preferentially used for oxidation by the TCA cycle in place of or in addition to carbohydrate since oxygen consumption increased during narcosis and RQ decreased indicating that TCA cycle

activity was not slowed and that greater fatty and amino acid catabolism occurred.

An apparently more profound narcosis induced by breathing 75% $N_2O$ at sea level did slow TCA cycle activity based on oxygen consumption but increased the amount of pyruvate found. The increased pyruvate could have been a function of increased glycolytic activity, decreased LDH activity or slowing of the TCA cycle and a buildup of its metabolic fuels. A greater percentage of glucose was oxidized in the TCA cycle although no apparent increase in glucose uptake occurred relative to surface controls. The decrease in plasma FFA and an increased RQ was interpreted as indicating a shift toward greater carbohydrate utilization at the expense of fatty acid oxidation.

Exposure to helium at 7 ATA caused an assumed moderate increase in metabolic rate but both glucose uptake and conversion to $CO_2$ were reduced at pressure. Glycolytic activity appeared to be about the same as control but there was greater conversion of pyruvate to lactate and increased plasma FFA levels at pressure. These data imply that lesser amounts of glucose were reaching the TCA cycle via glycolysis and that fatty acid metabolism may have increased as a result.

Thus, while a clear metabolic pattern characteristic of each environment is not evident, some general trends can be noted. At pressure, a general shift to a more lipid based metabolism at the expense of carbohydrate utilization is postulated. This trend was more evident in helium (non-narcotic) such that nitrogen (narcotic) may have had an attenuating influence. The increased metabolic rate seen at pressure was probably more a pressure effect than a gas-dependent one. Nitrous oxide narcosis, in the absence of increased pressure, decreased metabolic rate and shifted metabolism toward a greater carbohydrate utilization at the expense of fatty acids. These changes were probably a function of the inert gas, the lack of compression and the more profound degree of narcosis.

The long term implications of our results are not clear. The metabolic response to the different environments was relatively discreet and the changes were not of a nature which could prove pathological if they persisted. There was an obvious decline in plasma FFA and lactate in the $N_2O$ experiments: otherwise, the effects did not consistently vary with time of exposure.

With regard to the potential long term effects of exposure to these environments, it is pertinent to note that rats exposed to normoxic mixtures of helium or nitrogen at 7 ATA for 14 days were observed to lose large amounts of weight, although anorexia may have been a contributing factor (12). Similarly, a loss of weight in rats exposed to from 5-30 ATA of helium has been reported (3). In this latter instance, urinary carticosteriod excretion was significantly elevated suggesting that the weight loss could have been the result of a general stress response. It is likely that any detrimental action of long term exposure to pressure is primarily a function of stress rather than a specific gas effect. The metabolic changes which we observed in hyperbaric nitrogen and helium are consistent with and might well have been a function of a general response to stress. Conversely, the metabolic changes in $N_2O$ may have been a specific effect of the narcotic state induced by that gas.

We conclude that exposure to 7 ATA pressure causes an increase in metabolic rate and general shift toward oxidation of fatty acids at the expense of carbohydrate. If narcosis, induced by nitrogen, accompanies the compression, the degree of this shift in metabolism is lessened. Breathing 75% $N_2O$: 25% $O_2$ at sea level substantially depresses metabolic rate and causes a relatively greater utilization of carbohydrate for catabolism. It is likely that the metabolic changes recorded in hyperbaric nitrogen and helium were a generalized response to the stress of elevated pressure while those observed in $N_2O$ may have been specific to the narcosis produced by that gas.

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Table 1. Percent difference between mean values of all samples collected during pre-exposure control and experimental periods for surface control (SC), $N_2$-$O_2$ or air at 7 ATA ($N_2$), He-$O_2$ at 7 ATA (He), and $N_2O$-$O_2$ at one atmosphere ($N_2O$). Numbers in parentheses are the range of mean values obtained during the 12 sample periods for the experiments. The data represent the averages of 9 experiments with $N_2$-$O_2$ or air and two experiments each for He, $N_2O$ and surface control.

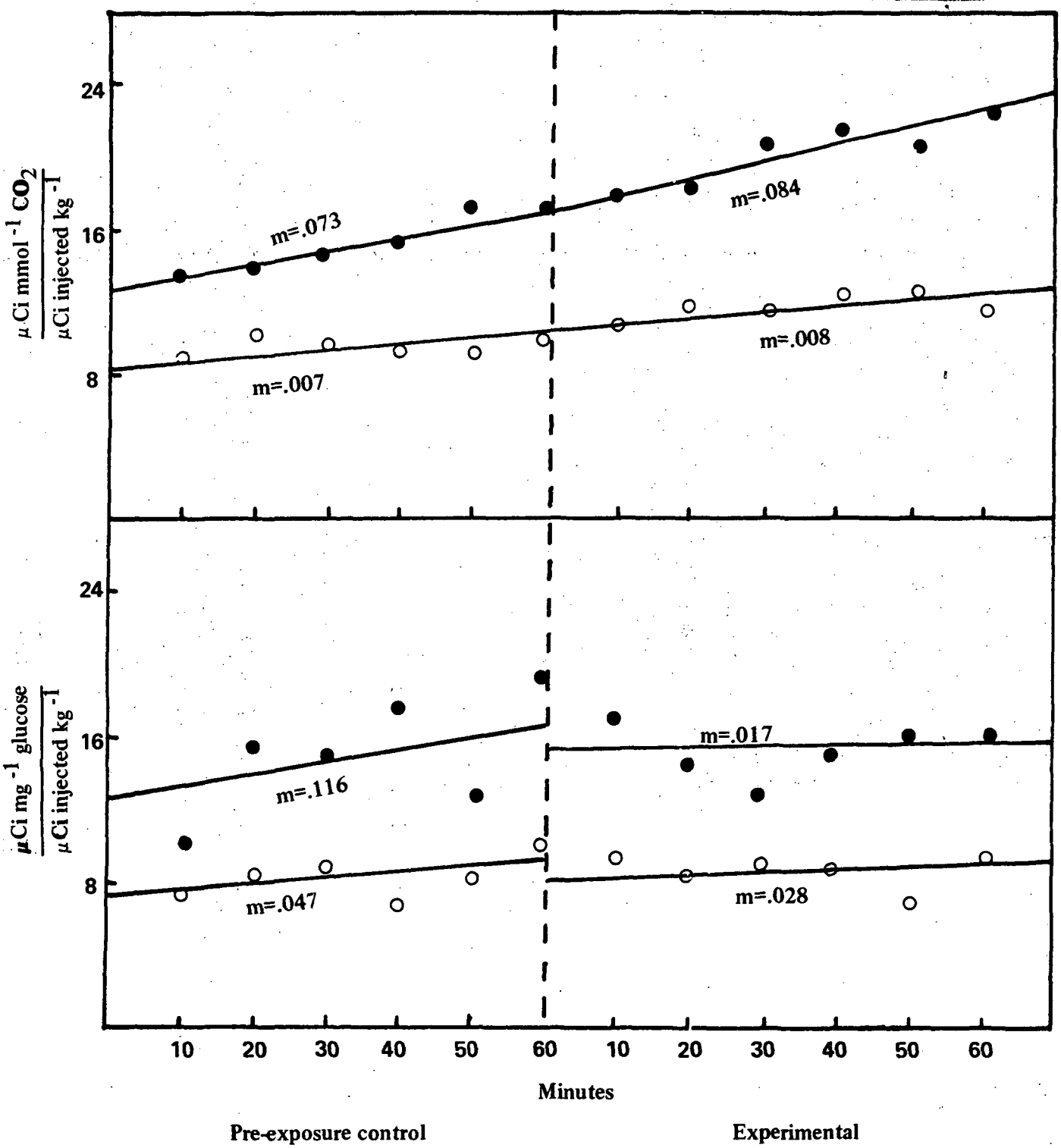
| Parameter | SC | $N_2$ | He | $N_2O$ |
|---|---|---|---|---|
| VER | | | | |
| $P_1$-$N_1$ | -1 | -32* | -16 | -13 |
| $N_1$-$P_2$ | -11* | -37* | -24* | +3 |
| EEG | | | | |
| $\alpha$ | † | -178* | +100 | -109 |
| $\theta$ | † | -78 | +72 | +114 |
| $\dot{V}O_2$, ml/kg/min STPD | 0 | +25* | ‡ | -43* |
| | (4.39-5.11) | (4.39-6.21) | (3.99-4.93) | (3.22-6.52) |
| $\dot{V}CO_2$, ml/kg/min STPD | -2 | +6 | +6 | +23* |
| | (3.76-4.28) | (3.79-4.29) | (4.31-5.95) | (5.22-6.66) |
| respiratory quotient | -2.6 | -15* | ‡ | +121 |
| | (.84-.89) | (.69-.93) | (.95-1.07) | (.82-2.06) |
| plasma glucose, mg% | -5* | +4* | +11* | 0 |
| | (248-272) | (182-196) | (165-190) | (252-272) |
| blood lactate, mg% | -7 | -10* | +16* | -12 |
| | (8.4-11.7) | (6.9-9.6) | (12.1-16.8) | (8.3-13.6) |
| blood pyruvate, mg% | +0.4 | -1.5 | +6 | +17* |
| | (.66-.82) | (.42-.56) | (.55-.92) | (.61-.88) |
| lactate: pyruvate ratio | -9 | -2 | +21 | -26* |
| | (12.7-18.0) | (14.1-20.0) | (10.2-20.6) | (11.6-20.8) |
| plasma FFA, meq/L | -19* | -8 | +15 | -45* |
| | (1.22-1.92) | (1.22-1.88) | (1.36-2.41) | (.59-1.86) |
| m, specific activity of expired $^{14}CO_2$ | +14 | +15 | -35 | +23 |
| | (.007-.008) | (.073-.084) | (.069-.108) | (.051-.063) |
| m, specific activity of plasma glucose | -30 | -85 | +20 | -25 |
| | (.028-.040) | (.017-.116) | (.020-.167) | (.006-.008) |
| rectal temperature, °C | -1.0 * | -0.8* | -0.3* | 0 |
| | (38.6-39.3) | (38.4-39.1) | (38.6-39.0) | (38.0-39.1) |
| skin temperature, °C | -1.0 * | -3.6* | -2.9* | +2.2* |
| | (35.9-36.5) | (33.3-35.3) | (34.8-36.2) | (35.5-35.9) |
| heart rate, bpm | +2 | -11* | -2 | +2 |
| | (143-175) | (134-162) | (143-174) | (116-160) |

* statistically significant at $P < .05$ or better

† data not collected

‡ data suspect due to mask leakage at pressure. Numbers in parentheses represent surface values only.

Figure 1. Mean specific activities of plasma glucose and expired $^{14}CO_2$ as a function of time for surface control ( ○ ) and hyperbaric nitrogen ( ● ) experiments. The lives represent a least squares regression analysis of the six sampling periods for the pre-exposure control and experiment periods. The slopes m were used to compare relative differences in the rate of change of specific activity during each period.


24
16
8
μCi mmol -1 CO2
μCi injected kg -1
m=.073
m=.084
m=.007
m=.008
24
16
8
μCi mg -1 glucose
μCi injected kg -1
m=.116
m=.017
m=.047
m=.028
10 20 30 40 50 60 10 20 30 40 50 60
Minutes
Pre-exposure control
Experimental