BIOCHEMICAL AND METABOLIC EFFECTS OF A SIX-MONTH EXPOSURE OF SMALL ANIMALS TO A HELIUM-OXYGEN ATMOSPHERE*

ROBERT W. HAMILTON, JR., JANIS D. COHEN,
GERALD F. DOEBBLER, LORENZO F. EXPOSITO, JOHN M. KING,
KENT H. SMITH and HEINZ R. SCHREINER

Ocean Systems, Inc., Research and Development Laboratory, Tarrytown Technical Center, Tarrytown, N.Y., U.S.A.

(Received 12 January, 1970)

Abstract. Rats and mice were exposed for periods of up to six months and two successive generations of mice were raised in a ground-level chamber system filled with 80% helium – 20% oxygen, at 24 °C. A duplicate chamber for controls contained a comparable nitrogen-oxygen mixture, and in both the other environmental parameters were well-controlled and nearly identical. Animals adapted to helium showed no greater increase in oxygen consumption ($P > 0.05$) when placed in helium-oxygen than did those raised in air. Growth rates were identical, but the helium mice consumed more food and water.

Selected biochemical analyses were made on the parent and two successive generations of mice. These included blood indices; electrophoretically separated tissue protein patterns from liver, skeletal muscle, and cardiac muscle; quantitative determinations of LDH, MDH, and G6PDH from the same tissues; serum insulin; and semi-quantitative histochemical estimates of liver glycogen. No cases of statistically significant difference or consistent trends were seen between the experimental environmental groups. Additional analyses of liver nucleotides and redox-coenzymes also failed to show a significant difference.

The relative weights of liver, heart, kidney, and diaphragm (wet and dry) were the same in both groups. Histopathological examination of kidney and adrenal tissue produced unremarkable findings and none that were attributable to the nature of the gaseous environment.

It must be concluded that prolonged exposure to helium-oxygen, relative to air, does not produce detectable changes in several key subcellular factors which might be altered by serious metabolic disturbances, and therefore the helium exposure is well tolerated.

1. Introduction

The search for the optimum atmosphere for long duration space flights and the current general acceptance of the two-gas concept has produced the suggestion that a mixture of helium and oxygen be used (Hargreaves *et al.*, 1966; Roth, 1965). Helium will probably offer advantages from an engineering point of view (Bonura *et al.*, 1967), and there is evidence that its peculiar physical properties will give the astronaut a slight decompression advantage (Roth, 1965; Hamilton and Schreiner, 1967). In any case, there are good reasons for renewed interest in the physiological effects of living in helium.

There is no reason to expect that helium can exert a strictly biochemical effect on

* Prepared under Contract No. NAS 2-3900 for Union Carbide Corporation, Linde Division Research Laboratory, Tonawanda, N.Y. for Ames Research Center.

living systems. Although compounds of heavier 'noble' gases have been produced (Allen, 1965), there are no known 'chemical' combinations involving helium. Inert gases do affect biological systems, examples of such action being diver's nitrogen narcosis (Bennet, 1966) and general anesthesia by xenon. These effects are attributed to weak intermolecular forces; they are not expected to be seen with helium (Schreiner, 1964). But the physical properties of helium are sufficiently different from those of nitrogen that possible environmental effects cannot be ignored. Different inert gases have different effects on growth rates of certain microorganisms (Schreiner *et al.*, 1962) and mammalian cell systems (Bruemmer *et al.*, 1967).

Exposure to helium has been shown to have measurable effects on higher animals. Small mammals show a consistent increase in metabolism as evidenced by an increase in oxygen consumption, carbon dioxide production, and food consumption (see Roth, 1965, for a review). This phenomenon seems to be due to the thermal properties of helium, since the increase is typical of the thyroxine-induced overdose that follows cold exposure (Leon and Cook, 1960). Further indirect evidence that heat loss is causing the helium effect is shown in a previous report from this laboratory (Hamilton *et al.*, 1966b) which reveals a convincing correlation between oxygen consumption and convective heat loss in small animals exposed to atmospheres having different thermal properties.

But that the 'thermal' explanation may not be the whole story is suggested by several reports on variations in oxygen consumption of isolated tissues as a function of the nature of the inert gas present in the incubator. South and Cook (1953) found evidence of such an increase under conditions where thermal properties of the gas are unlikely to be important. Succeeding investigations have not always agreed with these findings (Rodgers, 1966; Maio and Neville, 1966): the question of whether or not helium exerts an effect on metabolism at the cellular or molecular level has not been settled.

That helium-oxygen atmospheres can be tolerated by man has been well established in two extensive and well-documented experiments conducted at the USAF School of Aerospace Medicine (Epperson *et al.*, 1966; Hargreaves *et al.*, 1966). No prominent metabolic effects of helium were seen in either experiment, but temperatures were held at a comfortable level and inert gas partial pressures were low – these were fractional-atmosphere experiments to simulate practical spacecraft conditions.

Yet another experiment involving helium has left an area of doubt. In a previous unpublished experiment in this laboratory (Schreiner, 1964), tissues from mice raised in helium-oxygen were subjected to a broad but not thorough biochemical survey. Most analyses failed to reveal any definite changes that could be attributed to the helium, but in one instance, electrophoretic separation of heart muscle protein revealed a prominent unidentified band. Many of these electrophoretic bands reflect enzymes involved in metabolic pathways, and some of these enzymes may be involved in the metabolic increase induced by helium. This experience is indeed limited, based on a single experiment, but the signs are portentous and cannot be ignored; the experiments being reported here were aimed in part at the resolution of this issue.

Possible anomalies in heart muscle protein as a result of exposure to helium provide

a focal point, but it is pertinent as well to look at other metabolic factors which might be involved. To this end we conducted a six-month experiment in which mice from an original and two succeeding generations were acclimatized to and kept in either a helium-oxygen atmosphere at sea level pressure or a similar atmosphere in which nitrogen was the inert gas. Analyses included tissue electrophoresis, quantitative assay for metabolic enzymes, surveillance of various other metabolic and hematologic factors and a pathological survey. In addition, the effect of living in helium on the metabolic response to helium was assessed.

Also, two preliminary experiments were conducted on the response of poikilothermic animals to helium, since this type of animal might have been expected to respond in the opposite direction from one which increases its metabolism when cooled.

The work reported herein was conducted during the period 28 September 1966 to 27 September 1967.

2. Chronic Exposure to Helium-Oxygen and Nitrogen-Oxygen Atmospheres

A. SUMMARY

Three generations of mice were exposed continuously at ground level pressure to helium-oxygen (80:20) and nitrogen-oxygen (80:20) in two specially designed semi-closed controlled environmental chambers maintained at 24 °C. During the six-month period of this experiment one generation (F_1) of mice was born and raised and a second (F_2) generation was conceived, born, and raised in these environments.

Metabolic studies conducted on groups of female mice from each generation showed a statistically significant increase in food and water consumption of the helium-exposed animals but no concomitant increase in growth rate.

Biochemical analyses of several selected tissue enzymes and of electrophoretically separated tissue protein patterns for cardiac muscle, skeletal muscle, and liver were made on several animals from each generation (parent, F_1 and F_2) and from each experimental environment (N_2 and He). Semi-quantitative histochemical estimation of liver glycogen was made. Electrophoretic protein patterns were determined by disc electrophoresis on polyacrylamide gels at high pH (8.6) and on cellulose acetate strips at low pH (5.4). Quantitative enzyme analyses were made for representative dehydrogenases of major metabolic pathways: lactate dehydrogenase, malate dehydrogenase, and glucose-6-phosphate dehydrogenase.

In no instance were statistically significant ($P<0.01$) differences or consistent trends in any biochemical parameter observed between animal generations or experimental environmental groups. It must be concluded that prolonged exposure to helium-oxygen (relative to air) does not produced detectable changes in macromolecular tissue constituents (proteins) of mice at least in so far as could be demonstrated in the tissues and macromolecule components considered in this study.

In addition, Dr. Henry A. Leon of the Ames Research Center, National Aeronautics and Space Administration, compared plasma insulin levels in a number of mice of the

study with similar negative results. Adenine nucleotide redox-coenzymes, pyruvate and lactate levels in liver tissues of mice of the present study were determined by investigators at the Illinois Institute of Technology Research Institute. Their analyses produced no statistically significant differences in the levels of these parameters that could be attributed to chronic exposure to helium.

There were no significant differences in hematocrit, hemoglobin, and red blood cell counts between helium-exposed and nitrogen-exposed mice.

Histological examinations of kidney and adrenal tissues and liver glycogen stains produced unremarkable findings, and none that could be attributed to the nature of the gaseous environment. Likewise, no trend or consistent difference in the weights of liver, kidney, heart, or diaphragm could be discerned between the helium and nitrogen-exposed group of animals.

B. INTRODUCTION

Inert gases of the helium group, i.e. helium, neon, argon, krypton, and xenon, while evidently not essential to life, do manifest certain physiological activities when employed as the inert diluent of a breathing gas mixture. Narcosis, decreased responses to stimuli, alteration of metabolism, decreased oxygen-dependent sensitivity to radiation and altered rate of development are some of the effects that helium group gases are capable of producing in the intact organism (Cook and Leon, 1959; Rinfret and Doebbler, 1961; Featherstone and Muehlbaecher, 1963; Roth, 1965).

Most of the work previously done in the field of inert gas physiology has involved short-term exposures to inert gases under moderate to high pressures. However, the problem at hand is not one of short-term or high-pressure exposure, but rather the chronic effect of exposure to a helium-oxygen environment at ground-level pressure.

Helium has been shown to accelerate metamorphosis of the fruit fly, *Drosophila melanogaster*, and the meal worm, *Tenebrio molitor* (Cook, 1950), and a remarkable acceleration of oxygen consumption was seen in mice breathing an 80:20 mixture of helium and oxygen (Cook *et al.*, 1951). Leon and Cook (1960) demonstrated that the accelerated metabolism of rats exposed to a helium-oxygen environment was most likely due to the greater heat loss in the presence of helium since the magnitude of this response decreases as the temperature differential between the animal and its gaseous environment decreases. This was confirmed in subsequent studies conducted at our laboratory (Schreiner *et al.*, 1965). The degree of metabolic acceleration, in the presence of helium, also diminished when the standard metabolic rate of the animals was increased by a variety of means (Leon and Cook, 1960). Hamilton *et al.* (1966a) at this laboratory showed convincingly, for a variety of inert gas-oxygen environments at ground level and at reduced pressures, that metabolic rates of rats and rabbits tend to be a function of their relative convective heat loss into the gaseous environment. The questions which are then posed are whether (1) succeeding generations of animals will adapt to living in a helium-oxygen atmosphere, (2) the effects of helium are indeed purely physical, and (3) metabolic interactions take place on chronic exposure to helium that can reset a new and homeostatic metabolic rate.

Very little published evidence is available to document the chronic effects of exposure to helium at ground level. Chickens and rats maintained in a 79% helium – 21% oxygen atmosphere for three and two weeks, respectively, showed a depression of oxygen consumption on exposure to air to a level significantly below that of controls maintained in air. As expected, the helium-exposed animals showed an increase in metabolism as compared with air controls (Rhoades *et al.*, 1965; Weiss and Rhoades, 1965). Rats maintained in a 79% helium – 21% oxygen atmosphere for 24 days did not show changes in urine volume or water consumption but food intake was increased (Weiss and Rhoades, 1965). A 68-day exposure of four human subjects to an oxygen-helium atmosphere at reduced pressure (Hargreaves *et al.*, 1966) revealed no important physiological alterations. In that study a partial pressure of helium was maintained which may not have been high enough to modify the physical properties of the gaseous environment sufficiently to bring about such alterations. In our study, a helium partial pressure of approximately 600 mm Hg, and an environmental temperature of 24 °C were maintained. These conditions are known to produce the characteristic acceleration of metabolism associated with exposure to helium (Schreiner, 1965).

C. EXPERIMENTAL PLAN

1. *General*

The exposures involved in this experiment present a somewhat difficult ecological problem in that the presumably *inert* component of the atmosphere is the primary variable. Every effort had to be made, therefore, to screen out the effects of other environmental parameters of well-known physiological significance. To this end an appropriate group of control animals was included in the experimental plan to minimize environmental influences that are unrelated to the nature of the inert gas.

Our attempt to achieve this led us to design a pair of environmental chambers in which parallel groups of rats and mice could be maintained, so that all measurement could be made in duplicate on both control and experimental animals.

A total pressure of approximately one atmosphere was selected for two reasons. First, it is simpler and less costly to operate at one atmosphere, and this simplicity makes the maintenance of stable environmental conditions much easier. Also, exposure at reduced pressure (though perhaps more realistically simulating a real spacecraft atmosphere) reduced the partial pressure of the inert gas and consequently its relative importance as an atmospheric component.

The oxygen partial pressure selected was set to be as near to normal sea level as possible: 150 mm Hg was chosen as the optimum set point, with a band width of ± 20 mm Hg. This level of PO_2 gives these experiments an advantage over others designed to investigate inert gas effects, in that toxic aspects of oxygen need not be considered. In many other experiments, including one conducted at this laboratory, effects of slightly increased oxygen tension could not be separated convincingly from possible effects of helium, and indeed there may have been some synergism involved (Hamilton *et al.*, 1966c).

A temperature of 24 °C was selected arbitrarily for this experiment, and is not necessarily optimal. A higher temperature, around 29 °C, could have been used to minimize thermal effects of helium, but this would have interfered with the investigation of possible adaptations to the metabolic stimulation by helium. We recognize that, from the viewpoint of heat transfer, 24 °C does not represent the same environment in helium-oxygen as it does in air, but then that is a primary variable in the experiment.

Relative humidity, as measured, likewise may not strictly represent an identical environment in the two gases, but the range of 40–60% is broad enough and easily enough handled by both man and our experimental animals that we felt it was a good choice. Since humidity and helium both may affect thermal balance, we considered it important to maintain humidity in an innocuous range.

Cage conditions were intended to be typical of the standard methods of housing laboratory animals. The cages were chosen because they would fit into the chambers, and afforded an easy method of handling animal wastes that would be practical under the somewhat difficult conditions of isolation that were involved.

The animal population was, of course, of initial importance to the experiment. For mice, an inbred strain (Manor Swiss MF-1) was chosen and obtained pathogen-free. This we felt would minimize the possibility of an infection in one chamber confounding the result. Animals were acclimated to our laboratory conditions under strict isolation from all other animals which might not be free from infection. An inbred strain of mice was chosen to preclude differences between generations following sibling matings.

A standardized method of obtaining samples for analysis was worked out. Mice were taken from both chambers in equal numbers, and as a rule, the source was not revealed to the analysts until analyses were complete. As far as possible, sampling times were so adjusted that animals from succeeding generations were of the same age at necropsy.

Forty parent generation mice were randomly divided into eight groups of five each. Four of these groups were then randomly placed into each of two semi-closed controlled environmental systems collectively referred to as CES-4. This apparatus, which is described in detail in the report by Hamilton *et al.* (1969), contains two completely

TABLE I

Animal and chamber terminology

CES-4	Complete chamber system for experimental and control chronic exposure study at ground level pressure
Chamber A	Semi-closed environmental system containing 20% oxygen – 80% helium
Chamber B	Semi-closed environmental system containing 20% oxygen – 80% nitrogen
P Generation	Parent generation of mice – the only individuals not born in CES-4
F_1 Generation	First generation born in CES-4
F_2 Generation	Second generation born in CES-4

separate but identical chambers identified as Chamber A and Chamber B (cf. Table I and Figure 1). Offspring (F_1 generation) of matings in the P generation were randomly culled to forty individuals. Offspring (F_2 generation) of matings in the F_1 generation were randomly culled to forty individuals of either sex. This terminology is summarized in Table I.

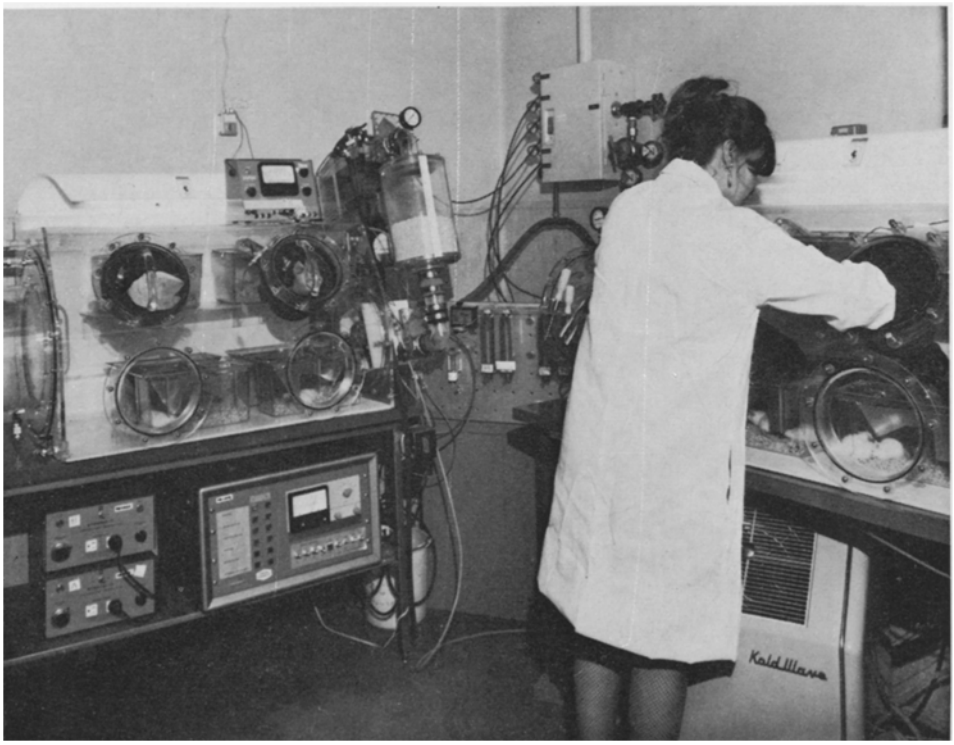

Fig. 1. Closed environmental system (CES-4). A dual chamber system for the chronic exposure of animals to potential spacecraft cabin atmospheres. Chamber A (helium-oxygen) is shown on left, chamber B (nitrogen-oxygen) at right. Master control unit for both chambers is shown beneath chamber A.

During the experimental exposure, a total of 160 F_1 mice and 60 F_2 mice were culled from the helium-oxygen environment (Chamber A), and 196 F_1 mice and 98 F_2 mice from the nitrogen-oxygen environment. The actual numbers may underestimate the actual population increase in view of known incidents of cannibalism among the experimental populations.

Both chambers of the CES-4 system were maintained at 24 ± 1.0 °C (gas temperature throughout the six-month experiment. Carbon dioxide was maintained within the range for 0.1 to 0.6% and nitrogen levels, particularly after the initial shakedown of the system, rarely exceeded 1% with an average value of about 0.5%. Relative humidity was maintained between 40 and 60% and an automatic light-dark cycle of 12 hours

was maintained throughout the experiment (lights on 7 a.m. – 7 p.m., lights off 7 p.m. – 7 a.m.) to achieve standard diurnal conditions.

Throughout this study, animals were randomly selected from the breeding colonies in Chambers A and B and were subjected to exsanguination and necropsy.

In addition to the parent generation of mice, six rats were placed in each chamber at the beginning of the experiment. They failed to breed in the chambers and were used for oxygen consumption studies after a six-month exposure.

2. *Food and water consumption, and growth*

Metabolic studies were conducted on five to ten female mice of each generation present in the two controlled environmental systems by placing them in separate cages where complete measurement of food and water consumption could be accomplished. Five animals each of the parent generation were placed in their metabolic cages and remained there for the duration of the experiment. Ten members of the F_1 generation were placed in each of the metabolism cages for a five-week period at which time the number of animals was randomly reduced to five. The F_2 generation was treated in the same manner as the F_1 generation. All animals in this study were weighed as a cage group twice weekly.

The metabolism cages were designed so that food and water consumption could be measured. Originally, $11\frac{1}{2}$ in. $\times 6$ in. $\times 6$ in. plastic cages were used; however, in order to measure food consumption, the cage covers had to be modified. The food-containing portion of the lid was covered with a piece of screening, attached in a hinge fashion. More food could be added easily by lifting the screen, but it could be closed to prevent any food from falling out of the feeders causing error in the consumption figure. Initially each screened cage cover was weighed when empty and then 300 grams of food (Rockland Rat Diet) was added to each food holder. Subsequent to the initial weighing, only pre-weighed amounts of food were added to each cage. The covers were re-weighed weekly and food consumption was calculated and expressed as grams of food consumed per gram animal per week.

Water was initially supplied by means of a baby bottle screwed into a metal cup-like cap ('chicken' type) from which the animals drank. Water level readings were taken daily; however, this arrangement soon proved to be cumbersome and inefficient because water loss introduced an inconstant error. The designed system to prevent water losses consisted of leaning the bottles in their normal, tilted position on the cage cover and replacing the caps with sippers containing ball bearing valves. This system minimized errors and allowed easy and accurate water measurement.

3. *Biochemistry*

During the course of operation of the environmental chambers, mice representing parent (initial population) as well as first and second generation animals born in the chamber environments, and all of approximately equal age (2 months), were removed at intervals. Animals were sacrificed by anesthetizing them with ether followed by exsanguination and removal of heart, liver, and skeletal muscle specimens for bio-

chemical analyses. Liver specimens were fixed and stained for glycogen (semi-quantitative) using the periodic acid-Schiff's technique (cf. Section 2.C.5). Livers, hearts, and skeletal muscle specimens were washed with cold saline, blotted, weighed, and aliquots homogenized at 0 °C with distilled water in glass Potter-Elvehjem homogenizers to give a 10% (*w*/*v*) homogenate.

Homogenates were centrifuged in a No. 40 rotor at 20000 rpm (26–36 000 × *g*) using a Spinco Model L preparative ultracentrifuge. Supernatant fractions were frozen in glass tubes in liquid nitrogen and stored at −170 °C. Just prior to use, supernatant fractions were thawed for quantitative enzyme analyses or were freeze-dried and reconstituted with water to a protein concentration of 100 mg/ml for electrophoretic separations.

Enzyme analyses included: lactate dehydrogenase (*L*-lactate: NAD oxidoreductase, IUB 1.1.1.27), malate dehydrogenase (*L*-malate: NAD oxidoreductase, IUB 1.1.1.37), and glucose-6-phosphate dehydrogenase (*D*-glucose-6-phosphate: NADP oxidoreductase, IUB 1.1.1.49). These enzymes, designated respectively LDH, MDH, and G6PDH, are key cellular enzymes of major metabolic pathways. LDH catalyzes the terminal glycolytic path reaction:

$$L\text{-lactate} + \text{NAD} = \text{pyruvate} + \text{NADH}_2;$$

MDH catalyzes the citric acid cycle reaction:

$$L\text{-malate} + \text{NAD} = \text{oxaloacetate} + \text{NADH}_2;$$

G6PDH catalyzes the hexose monophosphate shunt reaction:

$$D\text{-glucose-6-P} + \text{NADP} = \text{6-phosphogluconate} + \text{NADPH}_2.$$

LDH was assayed by measuring the decrease in pyruvate in the presence of NADH. Pyruvate was determined colorimetrically as the dinitro-phenyl-hydrazone (Sigma Chemical Company, Technical Bulletin on LDH and Wroblewski, 1957). One unit of LDH converts 4.8×10^{-4} μmoles pyruvate/min at 25 °C. MDH was measured as decrease in absorbance at 340 nm in a system of phosphate buffer, oxaloacetate, and NADH (Bergmeyer and Bernt, 1963); one unit is equal to an optical density (O.D.) change of 0.001/min. G6PDH was measured as the increase in absorbance at 340 nm in a triethanolamine buffer system containing NADP and D-glucose-6-phosphate (Lohr and Waller, 1963); one unit was equal to an O.D. change of 0.001/min.

Electrophoretic separations of soluble tissue proteins were effected on cellulose acetate strips (12 × 1 in.) and on polyacrylamide gels (discontinuous electrophoresis) using aliquots of reconstituted freeze-dried tissue homogenate supernatant fractions (100 mg protein/ml). Methods were those described in the Gelman Instrument Company technical instructions for Sepraphore III for cellular acetate electrophoresis and by Rendon (1965) for polyacrylamide disc electrophoresis, and are summarized in Table II.

TABLE II

Conditions for electrophoresis

	Cellulose acetate (Sepraphore III)	Polyacrylamide gel
Current	250 volts	2.5 m amp/tube
Time	5 hours	75 min
Sample	5 μl (500 μg protein)	2 μl (200 μg protein)
Staining	0.5% Ponceau S in 5% TCA, 5 min	Amido Black, 60 min
Destaining	Three times, 5% acetic acid	Electrophoretic destaining in 7% acetic acid 15 ma/tube, 90 min
Detection	Photovolt densitometer, wet, uncleared strips, 505 nm filter, response setting 5	Photographic, high contrast film

TABLE III

Enzyme summary

Supernatant fraction	Protein concentration mg/ml[d]	Liver homogenate enzyme activities, units/ml[e]		
		MDH	G6PDH	LDH
Crude[a]	24.5	1.65×10^5	2.73×10^3	3.96×10^4
Mitochondria-free[b]	9.2	1.53×10^5	3.10×10^3	3.92×10^4
True[c]	5.2	1.37×10^5	3.64×10^3	3.84×10^4

[a] Supernatant following centrifugation ($600 \times g$, 10 min).
[b] Supernatant following centrifugation ($8500 \times g$, 10 min).
[c] Supernatant following centrifugation ($18000 \times g$, 60 min).
[d] Determined by absorbance at 280 mμ relative to albumin.
[e] Homogenate: 10% wet weight tissue in distilled water, prepared with Potter-Elvehjem homogenizer at 0°C.

Preliminary studies demonstrated the adequacy of the homogenization, centrifugation, and assay methods to give a soluble tissue fraction of reasonably constant enzyme composition relative to the crude homogenate, and free of particulate matter (see Table III). Electrophoretic separations in each medium were done at pH 8.6 and 5.4. Optimum separation of bands was obtained using the gel disc electrophoresis at pH 8.6 with tris buffers (Rendon, 1965) (see Figure 2) and the cellulose acetate electrophoresis at pH 5.4 (0.2 M tris-Malate buffer).

4. *Hematology*

Exposure to operational spacecraft atmospheres has resulted in disturbances of the red blood cell system in chamber tests (Helvey *et al.*, 1965; Brooksby *et al.*, 1966) and spaceflights (Swisher and Fisher, 1966). It has been hypothesized that these hematologic effects were a result, at least in part, of increased levels of oxygen partial pressure.

Hamilton *et al.* (1966b) reported reduction in hematocrit, hemoglobin, red cell count, and red cell volume in animals exposed to helium-oxygen atmospheres for seven days. The possibility of a compensating erythropoiesis was suggested by the

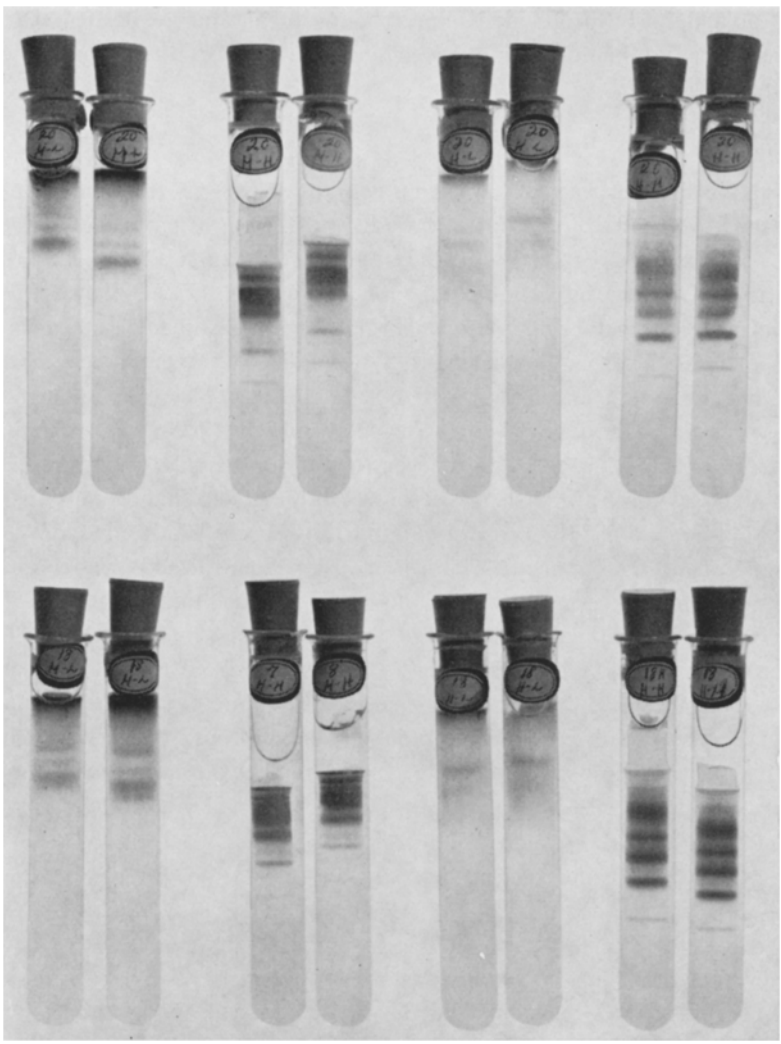

Fig. 2. Photographs of disc electrophoresis patterns of mouse muscle (M) or heart (H) extracts resolved under low (L) (pH 5.4) or high (H) (pH. 8.6) Ph conditions. The eight M tubes are on the left; H tubes to the right. The tubes with the darker, clearer patterns were done at high pH.

trends toward an increased reticulocyte count and greater iron uptake values. In disagreement with this hypothesis, these authors found no change in survival of injected donor red cells. Because of these earlier observations made in this laboratory, it was decided that hematocrits, red blood cell counts, and hemoglobin content should be determined.

Hematocrits were measured by the micro-capillary method and red blood cells were counted in an Improved Neubauer hemocytometer. Hemoglobin was measured by the cyanmethemoglobin method in which 5 ml of Drabkin's reagent were added to 0.020 ml of well-mixed whole blood. The optical density of the resultant mixture was de-

termined on a Band L Spectronic 20 Spectrophotometer and the result was translated into gram per cent of hemoglobin by use of a calibration curve based on an Acuglobin hemoglobin standard.*

5. *Pathology*

No pathological changes attributable to a helium-oxygen environment have been reported in the literature, but to our knowledge no exposures have been made for the duration accomplished in the present study. We therefore undertook histopathological examination of kidney and adrenal sections. In view of the fact that helium atmospheres increase the metabolic rate of mammals, it was also decided to measure liver glycogen content. The periodic acid-Schiff stain (PAS) was employed for this test, realizing that in most instances a positive reaction should be taken as denoting the presence of polysaccharides, glycoproteins or glycolipids (Lillie, 1954). Samples were stained with a PAS stain and classified as to the number of cells containing much glycogen and the staining intensity graded 1 to 4 of those cells that do contain glycogen.

Tissue samples for histological examination were preserved in alcoholic formalin, PAS samples in aqueous formalin. Gross necropsy was performed on all animals taken out of the chambers. An attempt was made to estimate the amount of body depot fat, but the confounding influence of sex, age, and size made this impractical. The entire liver, heart, left kidney, and diaphragm were dissected from randomly selected animals. These organs were washed with physiological saline, blotted, and weighed. The diaphragms were then dried at 96 °C to constant weight and this dry weight was recorded. Liver, hearts, and representative samples of skeletal muscle were subjected to biochemical analyses (cf. Section 2.C.3.).

D. RESULTS

1. *Food and water consumption, and growth*

The following general conclusions may be drawn from our results: parent generation helium mice, as well as mice born in the helium-oxygen environment (F_1 and F_2 generation), ate more food and drank more water but did not grow faster than animals raised in nitrogen-oxygen. The difference in the rate of water consumption was statistically significant only if the animals were not crowded (5 instead of 10 mice per cage).

Food consumption in grams of feed consumed per gram mouse per week, and water consumption in milliliters of water consumed per gram mouse per day were compared using the paired t-test (basis of pairing: date of measurement). Comparison was also made of data obtained with the metabolism cages containing both 5 and 10 animals to determine if the number of mice per cage affected the metabolic parameters. A statistical summary is given in Table IV.

Without exception the helium mice in all generations consumed more food than the nitrogen mice. This is illustrated in Figure 3. The F_1 and F_2 mice were reduced from

* Ortho Pharmaceutical Corporation, Raritan, N.J., U.S.A.

TABLE IV

Statistical summary of food and water consumption of mice maintained in chambers A and B

Comparison	t	df	P	Result
Food consumption: (gm/gm mouse/week)				
Parents He vs. N_2	7.380	15	<0.001	He mice $>$ N_2 mice
F_1, He vs. N_2	6.747	13	<0.001	He mice $>$ N_2 mice
F_2, He vs. N_2	4.245	5	<0.005	He mice $>$ N_2 mice
Water consumption: (ml/gm mouse/day)				
Parents, He vs. N_2	6.700	99	<0.001	He mice $>$ N_2 mice
F_1, He vs. N_2				
10 mice/cage	1.298	22	>0.05	no difference
5 mice/cage	14.943	69	<0.001	He mice $>$ N_2 mice
F_2, He vs. N_2				
10 mice/cage	1.136	37	>0.05	no difference
5 mice/cage	4.589	27	<0.001	He mice $>$ N_2 mice

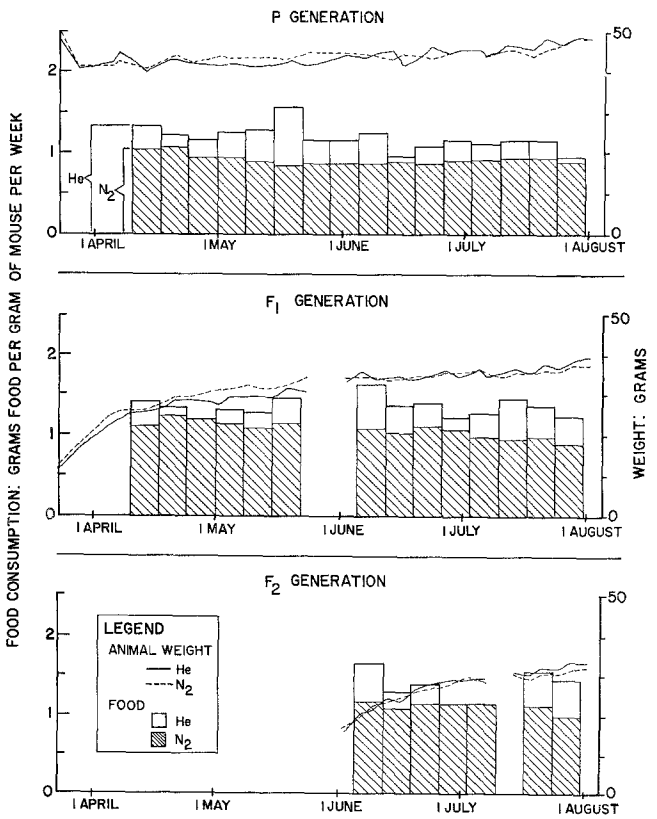

Fig. 3. Food consumption and growth of mice chronically exposed to helium-oxygen and nitrogen-oxygen at ground level pressure.

TABLE V

Mean values of food consumption

Generation	P		F_1		F_2	
Inert gas	He	N_2	He	N_2	He	N_2
Food consumption (gm/gm mouse/week)	1.19	0.913	1.35	1.06	1.60	1.12

The steady increase with generation shown here is due to the fact that the older generations were summated over a period of their life cycle when metabolism was lower.

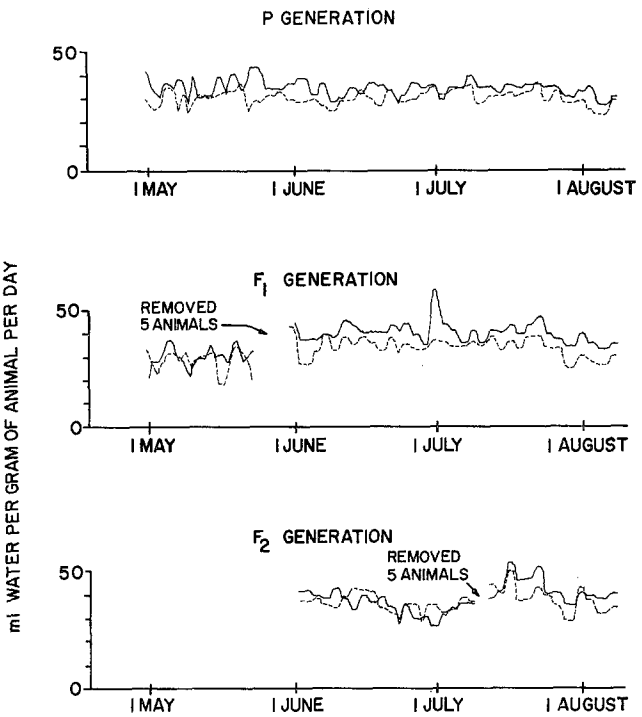

Fig. 4. Water consumption of mice chronically exposed to helium-oxygen and nitrogen-oxygen at ground level pressure. —— He, ---- N_2.

10 to 5 animals per cage at the breaks in the growth curves. Mean values are listed in Table V.

Throughout the experiment, the P generation animals in the helium chamber drank more water than did the control mice in the nitrogen chamber. The same observation was made with F_1 and F_2 generation mice when the animal population was five subjects per cage (Figure 4). With ten mice per cage, no statistically significant difference in water consumption could be seen between helium and nitrogen mice (Table IV). Mean values of water consumption $\pm$ one standard deviation and number of measurements (in parentheses) are given in Table VI for animal populations of five mice per cage. A statistical summary is given in Table IV.

TABLE VI

Water consumption, five mice per cage

Generation	P	
Inert gas	He	N_2
Water consumption (ml/gm mouse/day)	0.34 ±0.04 (94)	0.31 ±0.04 (99)
Generation	F_1	
Inert gas	He	N_2
Water consumption (ml/gm mouse/day)	0.41 ±0.07 (69)	0.34 ±0.04 (69)
Generation	F_2	
Inert gas	He	N_2
Water consumption (ml/gm mouse/day)	0.43 ±0.05 (25)	0.39 ±0.06 (27)

Reduction of the animal population from 10 to 5 in the F_1 and F_2 generation study resulted in an apparent increase in water consumption in both the helium and the nitrogen environments (Figure 4). This increase was greater for the helium mice than for the nitrogen controls.

The F_1 mice in the nitrogen chamber were on the average about 10% heavier than the helium mice when the cages contained ten mice each, a condition which lasted for 62 days. When the number of mice per cage was reduced to five, this difference disappeared. Even though the helium animals consumed significantly more food than the nitrogen animals, no systematic difference could be discerned in the rate of weight gain of the two animal populations (Figure 3).

2. *Biochemistry*

Dehydrogenase enzyme activities. We measured malate dehydrogenase (MDH), lactate dehydrogenase (LDH), and glucose-6-phosphate dehydrogenase (G6PDH) enzyme activities in the $26000 \times g$ supernatant fraction of aqueous homogenates of liver, cardiac muscle, and skeletal muscle taken from three generations of mice living in air (nitrogen-oxygen) and in helium (helium-oxygen). Results are presented in Table VII for the parent generation, pooled first and second generation offspring, and pooled total population groups and for the respective tissues and environments.

Considerable variation among individual animals for each enzyme of a particular tissue was found. Standard deviations on the average values for each group were usually 20–30% of the mean; in several instances (i.e. some LDH and most G6PDH) it was as great as 50% of the mean for a group. Where averages for different animal or environment groups appeared to differ, unpaired standard *t*-test statistical analyses were made. In no case were differences significant at the $P < 0.01$ level. In two instances differences significant at the $P = 0.05$ level were found. When the P-generation animal group in nitrogen was compared to the *P*-generation group in helium, MDH levels

TABLE VII

Quantitative dehydrogenase activities in mouse tissue homogenate supernatant fractions

Enzyme	Animal group	Nitrogen environment			Helium environment		
		Heart	Liver	Skeletal muscle	Heart	Liver	Skeletal muscle
MDH	P	1.45 ± 0.17(12)	0.94 ± 0.22(6)	0.22 ± 0.06(7)	1.29 ± 0.06(13)	0.90 ± 0.14(6)	0.22 ± 0.07(8)
MDH	$F_1 + F_2$	1.41 ± 0.22(6)	0.81 ± 0.16(7)	0.22 ± 0.08(6)	1.56 ± 0.29(6)	0.73 ± 0.14(7)	0.20 ± 0.05(6)
MDH	Pooled	1.44 ± 0.18(18)	0.87 ± 0.19(13)	0.22 ± 0.06(13)	1.38 ± 0.24(19)	0.81 ± 0.16(13)	0.21 ± 0.06(14)
LDH	P	2.81 ± 0.60(12)	1.62 ± 0.18(6)	2.43 ± 0.42(7)	2.74 ± 0.58(13)	1.85 ± 0.21(6)	2.62 ± 0.49(8)
LDH	$F_1 + F_2$	2.97 ± 1.07(7)	2.05 ± 0.46(7)	2.84 ± 0.30(8)	3.19 ± 0.58(7)	2.03 ± 0.29(7)	2.84 ± 0.32(8)
LDH	Pooled	2.86 ± 0.78(19)	1.85 ± 0.41(13)	2.64 ± 0.41(15)	2.90 ± 0.60(20)	1.95 ± 0.26(13)	2.73 ± 0.42(16)
G6PDH	P	514 ± 201(11)	2820 ± 1610(6)	274 ± 115(7)	581 ± 167(12)	1380 ± 947(6)	245 ± 116(8)
G6PDH	$F_1 + F_2$	594 ± 264(7)	2110 ± 1250(7)	313 ± 123(8)	640 ± 117(6)	1140 ± 202(7)	274 ± 97(8)
G6PDH	Pooled	527 ± 220(18)	2430 ± 1410(13)	294 ± 117(5)	601 ± 151(18)	1250 ± 640(13)	260 ± 104(16)

Values are averages ± 1 standard deviation.
Numbers in parentheses indicate numbers of individual animals used.
LDH = lactate dehydrogenase [(units/gm fresh tissue) $\times 10^{-5}$].
MDH = malate dehydrogenase [(units/gm fresh tissue) $\times 10^{-6}$].
G6PDH = D-glucose-6-phosphate dehydrogenase [(units/gm fresh tissue)].
P, F_1, F_2, and pooled refer to parent, first, second, and pooled generations of animals.
Because of limited numbers of animals in F_1 and F_2 groups, these were pooled as $F_1 + F_2$.

(but not LDH or G6PDH) were found to be higher in nitrogen (1.45×10^6 units/gm average) than in helium (1.29×10^6 units/gm) for heart tissue ($P < 0.05$). When the P-generation mice in helium were compared to the pooled $F_1 + F_2$ generation mice also in helium with respect to heart tissue MDH, significantly lower levels of the enzyme were found for the P-generation mice (1.29×10^6 units/gm) than for the pooled $F_1 + F_2$ generation mice (1.38×10^6 units/gm) ($P > 0.02$). In no other comparison of groups were differences found significant at $P \leqslant 0.05$.

From the pooled group data of Table VII, relative tissue dehydrogenase activities were calculated for respective tissue types. For LDH the decreasing order of relative activities was: heart (H) > skeletal muscle (SM) > liver (L); Wroblewski (1957) has reported the same order, H > SM > L, for human and animal tissue LDH. Meister (1950) and Dixon and Webb (1964) show relative LDH activities: SM > L > H. Tepperman and Tepperman (1965) discuss adaptive increases in liver enzyme activities and the recognized wide fluctuation in liver dehydrogenase activities.

For MDH our relative tissue order was: H > L > SM. This enzyme is usually considered to be mainly a mitochondrial enzyme, but its cellular distribution is known to vary widely with different organs. In heart it appears primarily in the cytoplasm. Relative tissue activities of the cytoplasmic enzyme according to Bücher and Klingenberg (1958) and Delbruch *et al.* (1959) are L > H > SM. Green (1936) (cf. Dixon and Webb, 1964) reported a relative order: H > SM > L. Therefore, there appears to be considerable disagreement among investigators in relative tissue distribution. Tepperman and Tepperman (1965) report wide fluctuation of MDH activity of liver under such control influences as hormones.

For G6PDH our relative tissue order was: L $\gg$ H > SM. The absolute activities of this enzyme considered on the basis of moles substrate converted per minute is low in most tissues, except red cells, mammary gland, and adipose tissues. Our relative tissue ranking is in complete agreement with that reported by Schmidt and Schmidt (1960) and Glock and McLean (1954).

We interpret these ranking data to indicate that our analytical methods are reliable

TABLE VIII

Relative dehydrogenase composition of different mouse tissues

Enzyme	Tissue			References
	Liver	Heart	Skeletal muscle	
Lactate dehydrogenase	43	30	100	Meister (1950)
Lactate dehydrogenase	62	100	90	This study
Malate dehydrogenase	19	100	38	Green (1936)*
Malate dehydrogenase	62	100	16	This study
Glucose-6-phosphate	100	37	11	Glock and McLean (1954)*
Dehydrogenase	100	22	12	This study

Values are relative to highest activity tissue for each enzyme taken as 100 (see Dixon and Webb, 1964).
* Data by these authors are for rat tissues.

and that they show no meaningful changes which might be attributed to the helium exposure.

In addition to qualitative ranking of relative tissue activities, we have calculated numerical relative tissue activities based on the most active tissue taken as 100. Our values are compared in Table VIII with corresponding results from the literature. Reasonably good agreement is obtained in almost all cases. Excellent agreement is obtained for G6PDH. In our analyses LDH of cardiac muscle was high (3 fold) relative to its ratio to LDH of other tissues as reported by Meister (1950) (cf. Dixon and Webb, 1964). This may reflect the method of killing our mice which involved ether anesthesia possibly confounded by anoxia. Conceivably this could have led to LDH release from injured anoxic cardiac muscle cells and its recovery at higher levels in a soluble supernatant fraction of the tissue.

Cellulose Acetate Electrophoresis. Soluble proteins (500 μg) were resolved on Sepraphore III cellulose acetate in tris-malate buffer (0.2 M) at pH 5.4. Migration from cathode to anode occurred in all cases. Bands at the origin (sample application region)

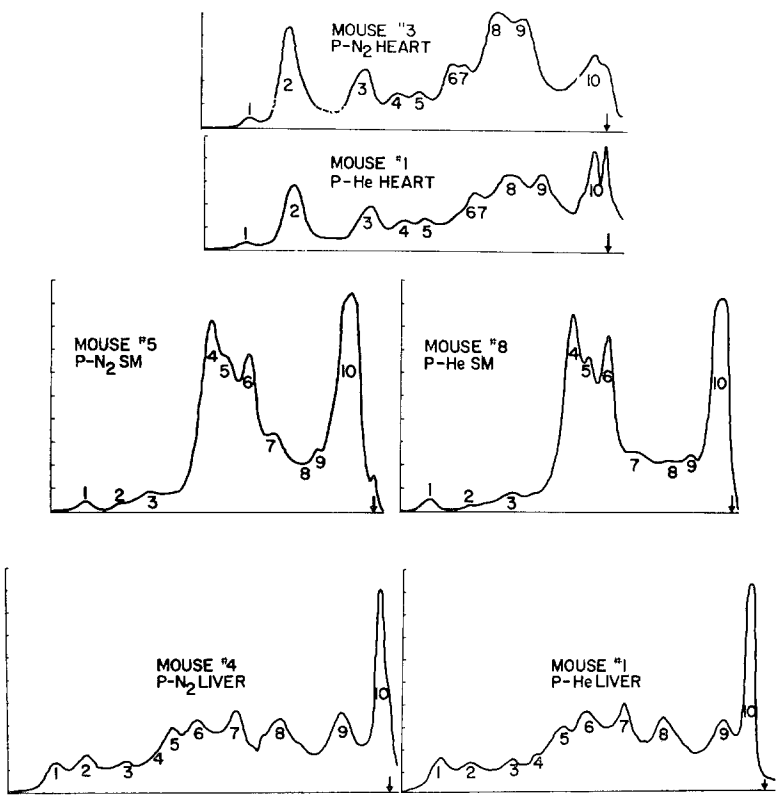

Fig. 5. Representative cellulose acetate strip electrophoresis patterns of proteins of mouse tissue extracts. Patterns are densitometer traces (arbitrary intensity scale) of actual strips. P refers to parent (initial) population; He and N_2 refer to animals from the helium-oxygen or nitrogen-oxygen environments respectively. Tissues are identified by name or for skeletal muscle (SM).

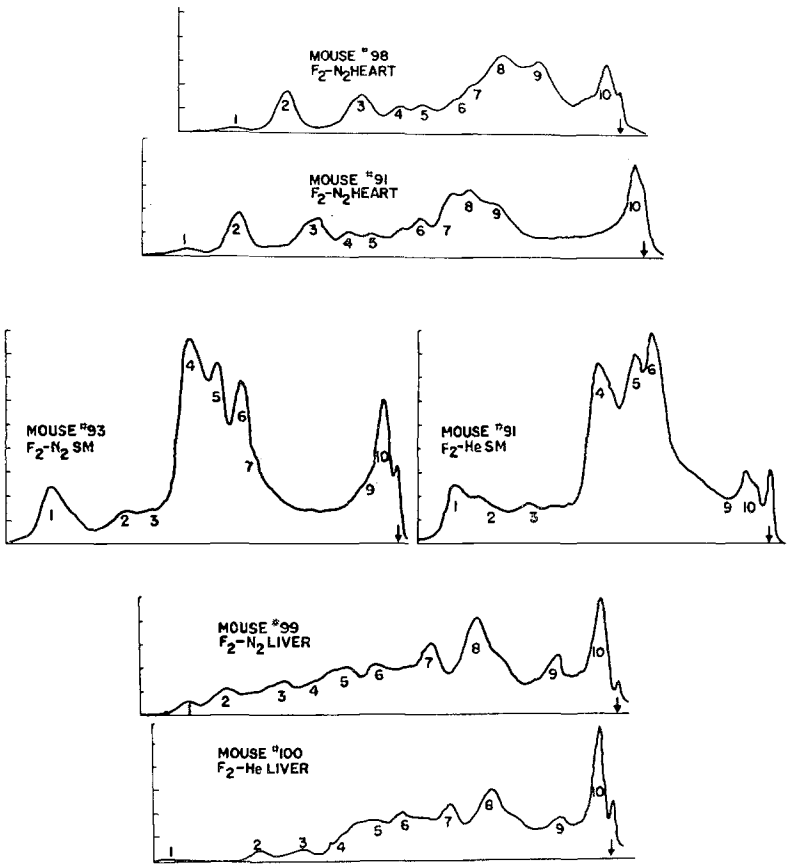

Fig. 6. Representative cellulose acetate strip electrophoresis patterns of proteins of mouse tissue extracts. Symbols and conditions are as in Figure 5. F_2 refers to second generation animals born in the chamber environments.

were almost always poorly resolved. Representative electrophoretic runs are shown in Figures 5 and 6 as densitometer traces for parent and F_2 generation animals. Within each figure are shown patterns for heart, skeletal muscle, and liver for animals from each environmental group.

For heart ten apparent bands, readily distinguished visually, were resolved in almost all specimens regardless of source (i.e. animal or environmental group). Densitometer traces are numbered according to positions of visually recognizable bands. Numbering is from No.1 for the fastest moving band (+end of strip) to No. 10 at the sample application origin (cathode). Numbers therefore decrease in the order of increasing mobility. Migration usually totaled 7.5–8.0 cm in the five-hour period used and at pH 5.5 all proteins resolved were apparently net negatively charged (i.e. albumin, α-or β-globulin-like relative to serum proteins). Under comparable conditions, serum albumin migrated 7.7 cm in five hours while γ-globulin remained near or within 1 cm of the origin. Among the resolved heart protein components other than the band (No. 10) at the

origin, two strong (Nos. 8, 9) and two moderate (Nos. 6, 7) bands appeared in the slow-mobility third of the strip; three moderate to weak bands (Nos. 3, 4, 5) occurred in the middle third of the strip; one strong band (No. 2) and one weak band (No. 1) appeared in the fast mobility third of the strip. The sum of bands Nos. 8 and 9 appeared to account for about 30% of the total protein; bands two and ten each contributed about 15% of the total. All bands were observed on all P-mouse heart extracts. The intensities of bands 2, 8, and 9 decreased markedly in our F_2 mice hearts over the

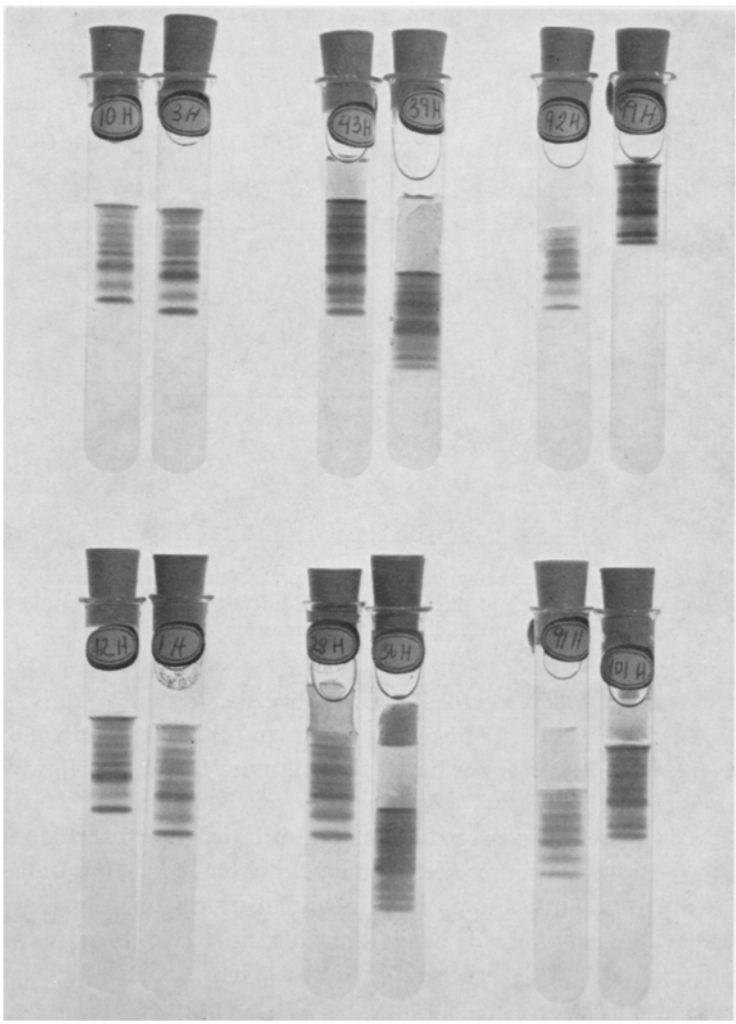

Fig. 7. Photographs of disc electrophoresis patterns of proteins of mouse heart extracts. Top row: animals from nitrogen-oxygen environment; bottom row: animals from helium-oxygen environment. From left to right in each row are pairs of animals from the parent (initial P), first (F_1), and second (F_2) generations.

corresponding P-mice hearts. Intensities of these bands were about half that of the corresponding bands of the P generation.

For liver again up to ten bands were resolved at least partially. There was an intense band near the sample origin but apparently with definite migration from the application site (very low mobility). Three moderate bands were present in the fastest third of the patterns; one moderate and three strong bands in the middle third; and two relatively strong bands, plus the very strong near-origin band in the slow third. Bands Nos. 8, 9, and 10 each constituted 15–20% of the total protein; bands Nos. 5, 6, and 7 con-

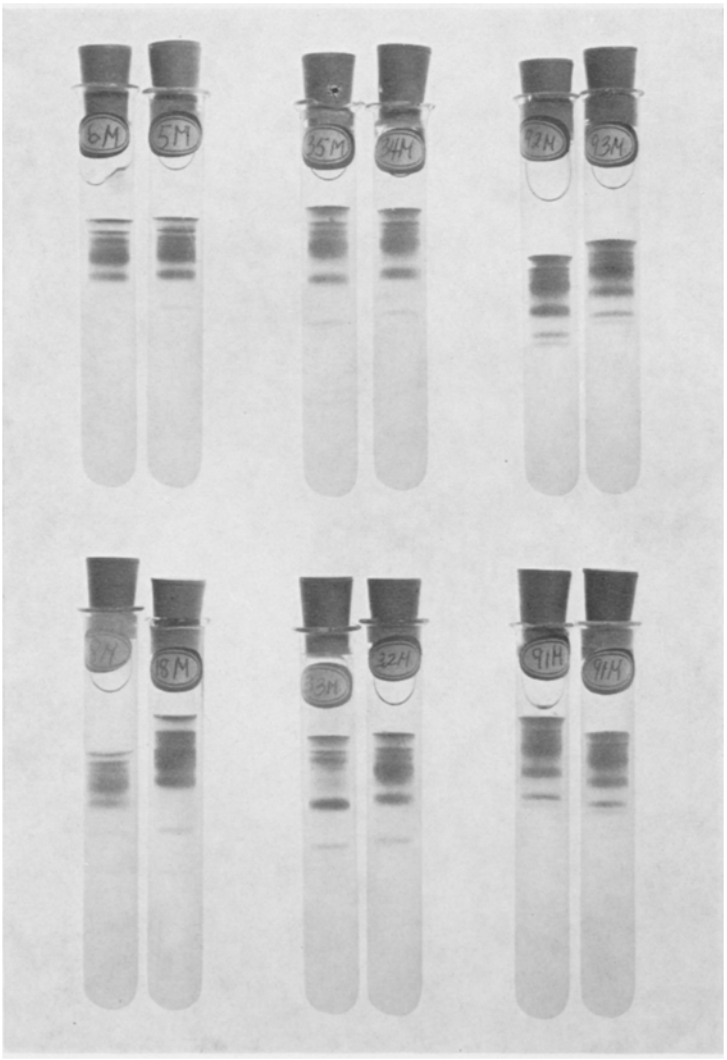

Fig. 8. Photographs of disc electrophoresis patterns of proteins of mouse skeletal muscle extracts. Arrangement of gels as in Figure 7.

stituted each about 10% of the total. The electrophoretic patterns of the F_2 group livers showed in general a decrease of up to 50% in the intensity of the No. 10 band (near-origin band) and an increase in intensity of the No. 8 band.

For skeletal muscle, again up to ten components were resolved. Three very weak bands occurred in the fast third of the pattern; four bands (three strong, one moderate) occurred in the middle third of the patterns. Bands Nos. 7, 8, and 9 were usually very poorly resolved. Bands Nos. 4, 5, and 6 together constituted over 50% of the total applied protein. Because of relatively poor resolution skeletal muscle could be considered as actually contributing five readily recognizable bands plus the band at the

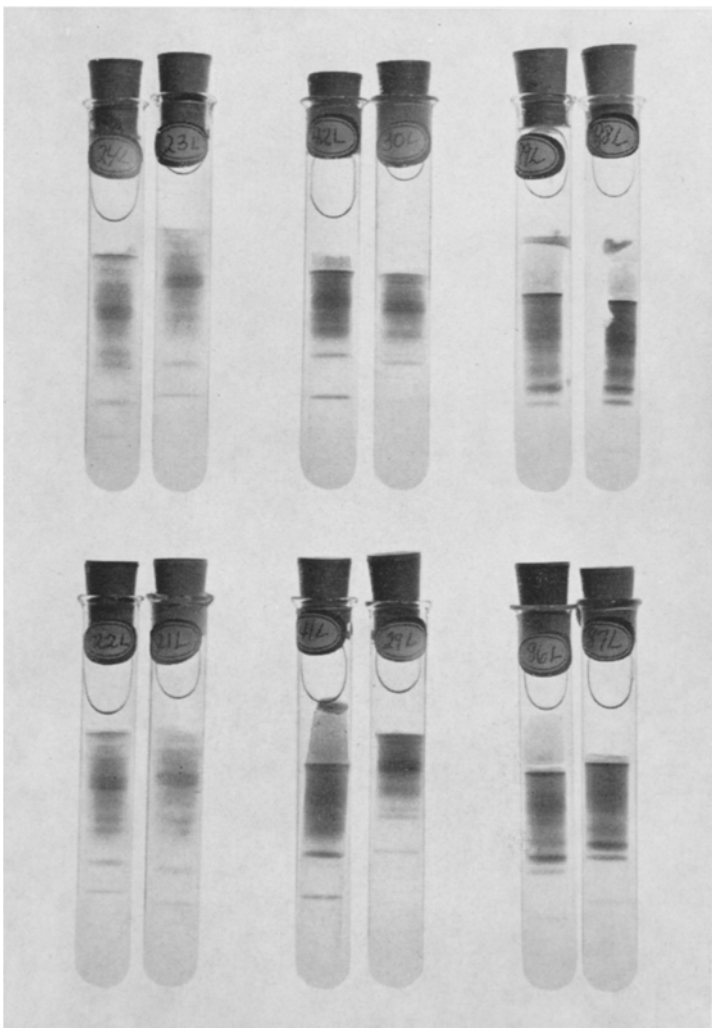

Fig. 9. Photographs of disc electrophoresis patterns of proteins of mouse liver extracts. Arrangement of gels as in Figure 7.

origin. In patterns for muscle of the F_2-mice the band at the origin decreased generally in intensity to half that of the P-group level. Bank No.1 increased several fold usually over the corresponding P-group valve. Relative intensities of bands Nos. 4, 5, and 6 were not constant among individual animals but varied widely. Variation was, however, apparently independent of the environmental gas group as a parameter.

Disc polyacrylamide gel electrophoresis. Fractionation of 200 μgm aliquots of soluble tissue proteins on polyacrylamide gels at pH 8.6 resolved up to 14 components from heart, 7 from skeletal muscle, and 11 from liver. Band intensities, and to a lesser degree, total numbers of resolved bands differed among individual animals. No correlations appeared to exist relative to animal groups or environmental gas groups. Representative gels are shown in Figures 7 (heart), 8 (skeletal muscle), and 9 (liver). Data were recorded photographically on high contrast film and actual gels and photographic prints were inspected visually; in several instances gels were scanned with a Photovolt Corporation densitometer. No attempt was made to quantify the gel protein patterns since differences among individual animals were great enough to preclude any reasonable chance of detecting correlations to the parameters of animal generation or environmental gas.

Insulin. In a supplementary study carried out by Dr. H. Leon, Ames Research Center, National Aeronautics and Space Administration, plasma insulin analyses were made on mice taken from the two environmental chambers. Insulin levels (units/ml) were 18 ± 10 (average $\pm$ 1 SD) for 16 mice from the helium environment and 17 ± 10 for 15 mice from the nitrogen environment. It is apparent, therefore, that no significant differences between the experimental groups existed with respect to this hormone parameter. Ranges for insulin levels in the two groups were 6–36 units/ml for the helium mice and 5–42 units/ml for the nitrogen mice. Considerable variation among individual animals, independent of the environmental exposure conditions, were noted as indicated by the ranges and standard deviations about the means which were encountered.

3. *Hematology*

The results of the hematological studies in general did not concur with previous studies in this laboratory or with other authors, as there were few remarkable changes. The red blood cell count in the parent generation was the same for the helium and nitrogen animals. Succeeding generations also showed no demonstrable differences in erythrocyte counts. Mean values with standard deviations and t values are given in Table IX. The absence of significant differences in hematocrit in any of the generations reconfirms the absence of significant differences in erythrocyte counts, assuming that the plasma volume remained the same. Hemoglobin values for helium mice of all three generations did not differ significantly from control values obtained from nitrogen animals.

4. *Pathology*

Histopathological examinations of kidney samples revealed a few round cell foci but

TABLE IX

Comparison of hematological indices of mice chronically exposed to helium-oxygen and nitrogen-oxygen at ground level pressure

	Hematocrit %		Hemoglobin g/100 ml		Red blood cells $10^6/mm^3$	
	P generation		P generation		P generation	
	He	N_2	He	N_2	He	N_2
$\bar{X}$	45.6	45.5	13.9	14.1	9.4	9.3
S	±2.2	±2 7	±0.9	±2.5	±0.3	±0.6
N	11	9	10	11	4	4
t	0.322		0.985		0.285	
	F_1 generation		F_1 generation		F_1 generation	
	He	N_2	He	N_2	He	N_2
$\bar{X}$	43.9	43.0	13.5	14.0	9.0	8.7
S	±1.8	±2.9	±1.8	±0.6	±0.7	±0.8
N	10	8	10	8	3	4
t	0.628		0.550		0.1656	
	F_2 generation		F_2 generation		F_2 generation	
	He	N_2	He	N_2	He	N_2
$\bar{X}$	45.4	45.5	14.8	14.8	8.1	9.1
S	±1.7	±2.5	±1.0	±0.7	±0.9	±0.9
N	10	10	10	10	4	4
t	0.1442		0.4225		1.092	

$\bar{X}$ – arithmetic mean.
S – standard deviation.
N – number of determinations.

these were not limited to any particular generation or gaseous environment. However, their presence was more frequent in the P generation.

Adrenal gland examination of the parent generation revealed large numbers of vacuoles and vacuolated cells at the cortical medullary junction. This finding is unique and is of unknown significance. In addition, there were many foam-type cells in this same area which have been seen in other aged mice by the animal pathologist participating in this study (J. M. King). It seems as if these foamy-type cells in the adrenal gland are multi-nuclear phagocytes undergoing some type of hydropic change.

Adrenal and kidney sections in the F_1 and F_2 generations appeared uniformly not remarkable. Some cuboidal linings were seen in kidney glomeruli in the F_2 generation but the significance of this finding is obscure at this time.

Sixteen liver specimens representative of the parameters of generation and environment were examined histochemically for glycogen. The semiquantitative data are shown in Table X. Except for an apparent low liver glycogen content for all F_1 generation mice, no correlation of glycogen level to the nature of the environmental gas or the length of exposure were noted.

TABLE X

Histochemical detection of relative glycogen content of mouse liver tissues

Animal No.	Generation	Experimental environment	Relative glycogen content
14	P	N_2	++++
15	P	N_2	++++
13	P	He	+++
16	P	He	++++
34	F_1	N_2	++
35	F_1	N_2	+++
38	F_1	N_2	++
39	F_1	N_2	−
32	F_1	He	−
33	F_1	He	+
36	F_1	He	++
37	F_1	He	+
92	F_2	N_2	++++
93	F_2	N_2	++++
90	F_2	He	++++
91	F_2	He	++++

Mean organ weights of female mice randomly selected from the P, F_1 and F_2 generations are shown in Table XI. Visual inspection shows no consistent trends in the values presented that can be ascribed to either the generation or the gaseous environment. Statistical evaluation of differences in organ sizes of animals maintained in either helium or nitrogen is not warranted.

E. DISCUSSION

Replacing the normal air atmosphere with a helium-oxygen atmosphere changes the oxygen utilization per unit of tissue. This has been described previously by numerous authors (Cook, 1950; Cook and Leon, 1959; Schreiner, 1964; Schreiner *et al.*, 1965; Hamilton *et al.*, 1966b; Rhoades *et al.*, 1966; Weiss and Rhoades, 1965). This observation was also made in the present study (cf. Section 3, below), which demonstrated that living in a helium environment, even through several generations, did not alter this effect. Food and water consumption remained increased throughout the experiment in the helium animals in all generations. The significant increase in water consumption in the helium environment is not unexpected. In normal mammals some 25 to 30% of the obligatory water loss occurs by way of the expired air. With an increased metabolic rate and increased oxygen consumption, one necessarily sees an increased respiration rate which does, in effect, cause an increased body water loss. Increases in the consumption of food materials demand a larger water intake, too, in order to maintain the normal electrolyte and fluid balance. Consequently, the water consumption increase conforms

TABLE XI

Organ weights of female mice chronically exposed to helium-oxygen and nitrogen-oxygen at ground level pressure

	Liver		Kidney		Heart		Diaphragm			
					P generation		Wet		Dry	
	He	N_2	He	N_2	He	N_2	He	N_2	He	N_2
$\bar{X}$	5.41	5.8	0.64	0.57	0.46	0.44	N.D.	N.D.	N.D.	N.D.
S	±0.56	±1.04	±0.03	±0.08	±0.03	±0.02				
N	10	10	6	6	10	10				
					F_1 generation					
$\bar{X}$	5.44	4.73	0.66	0.54	N.D.	N.D.	0.35	0.31	0.09	0.08
S	±0.67	±0.44	±0.06	±0.03			±0.04	±0.10	±0.01	±0.01
N	6	7	5	5			6	7	5	5
					F_2 generation					
$\bar{X}$	5.17	4.77	0.58	0.56	0.44	0.46	0.37	0.38	0.09	0.09
S	±0.45	±0.15			±0.06	±0.03	±0.02	±0.05	±0.01	±0.01
N	4	4	2	2	4	4	4	4	4	4

$\bar{X}$ – arithmetic mean.
S – standard deviation.
N – number of measurements.
All organ weights are expressed as % of whole body weight.

to our expectations. The important corollary of these measurements is that metabolically the F_2 generation appeared the same as the P generation, or as animals that have only been exposed briefly in previous studies. One possible explanation for the increase in water consumption seen when the animal population set aside for metabolic studies was reduced from ten to five is that these animals increased their physical activity in the relatively uncrowded condition (there was, however, no commensurate increase noted in the food consumption of these animals). The less crowded cage possibly had a lower relative humidity.

The rate at which both experimental populations gained weight in all three generations was not significantly affected by the nature of the gaseous environment. Similarly, there was no discernible effect on organ weights or on hematological indices that could be attributed to the helium exposure. While the results of the pathological examination of liver glycogen and kidney and adrenal tissue also failed to show up an effect of the helium exposure, the round cells that were detected in kidney specimens have been shown to be characteristic of the more advanced stages of acute or chronic inflammation (Stengel and Fox, 1915), and may therefore represent an indigenous pathology of the animal colony related to confinement *per se*.

Biochemical analyses of selected dehydrogenase activities and of electrophoretically separated protein patterns of cardiac muscle, skeletal muscle, and liver tissues demonstrated no differences in any instance between animals exposed to the nitrogen-oxygen compared to the helium-oxygen environment. With respect to dehydrogenase activities (LDH, MDH, or G6PDH) no differences significant at the $P<0.01$ level were found among animal generation groups or environmental gas groups. Heart MDH levels in the helium P mice were lower than in the corresponding nitrogen animals, but were increased in the F_1+F_2 generations raised in helium-oxygen ($P<0.05$). Distinct differences, as expected, occurred among different tissue types. Electrophoresis patterns of proteins exhibited some differences attributable to mice generation group differences but not to environmental gas groups. Wide variation among individual animals, independent of experimental parameter groups, occurred in dehydrogenases and in electrophoretic protein patterns.

Tissue enzymes and protein patterns of normal animal tissues and of tissues from tumor-bearing animals and animals in particular metabolic or disease states have been determined by many investigators. Glock and McLean (1954) have reported levels of the direct oxidative pathway of carbohydrate metabolism in various normal mammalian and tumor tissues. Frenkel (1965) among others has reported enzyme patterns for heart supernatant fractions. Hartshorne and Perry (1962) and Lauryssens and Lauryssens (1964) have described, respectively, electrophoretic patterns of adult and foetal-rabbit muscle, and mouse and human normal and dystrophic muscles. Dixon and Webb (1964, pp. 638–43) summarize a variety of tissue and species enzyme patterns.

With respect to our dehydrogenase enzyme measurements, relative tissue activities agree reasonably well with previously reported relative tissue distributions. We appear possibly to be high with respect to cardiac muscle (heart) lactate dehydrogenase in all our animals. This may, as discussed in Section 2.D.2, reflect the method of killing our

mice, which involved ether anesthesia. Our determinations of glucose-6-phosphate dehydrogenase, which yielded low absolute values on all tissues and especially low values on heart and skeletal muscle, are in excellent agreement with previously reported tissue distributions for this enzyme and also serve to document the essential absence of any significant blood contamination of our heart and skeletal muscle tissue. Any blood contamination, by virtue of high red cell G6PDH levels, would have shown up as abnormally high tissue G6PDH values.

In an earlier unpublished study conducted in our laboratory (Schreiner, 1964), elctrophoretic (cellulose acetate) separation of heart proteins of mice raised in helium-oxygen showed evidence of an additional or enhanced rapid-mobility protein component not observed in extracts of hearts of control mice raised in air. In view of our present more extensive electrophoretic patterns study which does not show differences between heart proteins of helium and air-exposed mice, it has appeared worthwhile to consider carefully the probable basis of the earlier finding.

Two explanations appear likely. The earlier work did not include a 'marker' determination of any component which could definitely rule out the presence of variable contamination of tissues by blood and plasma proteins. In the current study our measurements of G6PDH does this. The fast mobility band found in earlier electrophoresis patterns of pooled mouse heart extracts is also identical in mobility with serum albumin and could have represented random or uncontrolled contamination of the helium group heart extracts with blood plasma.

A second possible interpretation centers upon the fact that the earlier study involved normally nonanoxic conditions of sacrifice of the mice (neck fracture) and a more-or-less incomplete initial homogenization process (Virtis blender). If by chance one or more of the mice of the helium group pool had become grossly anoxic, there would have been a release of cardiac LDH isoenzymes which would have been readily extractable even with incomplete cellular disruption. The major cardiac LDH isoenzyme band has high mobility and in high concentration likely could have been detected on cellulose acetate by protein stains. This band would have been in the same region of the electrophoretic patterns in which the helium-related component was observed. While these interpretations have not been subjected to controlled experimental verification, it should be readily feasible to do so, if despite the results of the present study the question of the earlier preliminary observations is still considered by anyone to be of importance.

In the biochemical studies, we have concentrated upon measurements of macromolecular composition – in particular of selected key metabolic pathway enzymes and soluble protein patterns. During the course of the environmental runs, mice were also provided to investigators of the Illinois Institute of Technology Research Institute who analyzed adenine nucleotides, redox-coenzymes (NAD, NADH, NADP, NADPH), pyruvate, and lactate in liver tissues. Together, these complementary studies, while limited in scope, should have allowed detection of major irreversible or steady-state compositional changes in metabolic pattern components of the animals involved. No such changes were observed.

Estabrook (1965) has discussed the limitations on interpretation of enzyme (and substrate) patterns since at best they represent nonphysiologic states extrapolated to the cell, almost always involve assay under nonphysiologic conditions devoid of *in vivo* activation and inhibitor complications, and reflect kinetics in an artificial homogeneous environment rather than the often compartmentalized system found *in vivo*. However, Tepperman and Tepperman (1965) have reviewed well-documented examples of "adaptive increases in enzyme activities of liver occurring along specific metabolic pathways when there is a large and sustained increase in substrate traffic through them". They point out that the dehydrogenases of the direct oxidative pathway (i.e., G6PDH) and the NADP-dependent malate enzyme are known to fluctuate widely in liver. Hormones can influence the observed activities of the hexose monophosphate shunt dehydrogenases (i.e., G6PDH) and MDH differentially.

Hess and Brand (1965) have discussed the possible types of energy metabolism control in terms of enzyme and metabolite profiles. The activity of enzymes can vary in response to the levels of substrates and products and by allosteric activation and inhibition through control metabolites. The concentration of enzymes can vary as a result of the operation of induction, repression, and de-repression process which involve genetically controlled protein synthesis.

In the biochemical measurements of tissue protein patterns and selected dehydrogenase enzymes of this study and of adenine nucleotides, redox-coenzymes, and selected metabolic intermediates determined on our experimental animals by investigators at the Illinois Institute of Technology Research Institute, attention has been given to the conventional but restricted view of metabolism as a steady state or equilibrium process. But it is becoming increasingly recognized that the metabolism of an organism and, in particular, the response of its metabolic patterns to environmental parameters, hormones, biophysical stimuli, etc., can involve transient responses of enzymatic systems. The analysis of the dynamics and control mechanisms of multi-enzyme systems and of the multiple interlinked pathways of energy metabolism has only recently been approached (Higgins, 1965) even theoretically. It is technologically unfeasible at this time to consider such a level of analysis of the metabolic response of an intact mammal to an environmental compositional variable such as the nature of the inert gas to which it is exposed. While it is certain that there is a molecular basis to the mammal's elevation in oxygen consumption in helium, this may well involve a metabolic control mechanism and a transient change in the dynamics of enzyme processes of respiratory metabolism. It appears likely that the increased oxygen use is not accompanied by irreversible metabolic changes or altered biochemical composition of tissues.

3. Effect of Helium on Oxygen Consumption of Animals Adapted to a Helium-Oxygen Environment

A. SUMMARY

In this study, comparative oxygen consumption measurements were made on female rats and mice adapted to and maintained in 80% helium – 20% oxygen for over six

months at approximately one atmosphere. Rats and mice from the same strains and generations comparably maintained in 80% nitrogen – 20% oxygen in an identical chamber were used as controls. This study included mice from the second generation born in the chambers.

As expected, the rate of oxygen consumption at 23.5 °C was significantly higher in helium-oxygen (80:20) than in air and this was the case regardless of whether the previous gaseous environment to which the animals had been exposed contained nitrogen or helium. There was no statistically significant difference (at $P=0.01$) observed in the magnitude of oxygen consumption in helium-oxygen or in air between the helium maintained animals and their nitrogen maintained controls. In no case was it possible to discern within acceptable statistical probability a metabolic acclimation of either rats or mice to chronic exposure to helium-oxygen.

Second generation animals conceived, born, and raised in either helium-oxygen or nitrogen-oxygen showed higher rates of oxygen consumption (per gram of body weight) in both helium-oxygen and air than their parent generation animals which had previously been exposed for six months to either of the two gaseous environments. This difference was statistically significant in two of the four experimental permutations examined, and is most likely a function of the age of the animals.

To determine if there are effects of acute exposure to helium that do not depend on the thermal properties of helium, oxygen consumption studies were conducted in air and in helium-oxygen on boa constrictors, a heterothermic ('cold-blooded') species. Preliminary results indicate a higher rate of oxygen consumption by these reptiles in air as compared with helium-oxygen at 23 °C and at 33 °C, providing further evidence for the thermal mechanism for the effect of helium on metabolism.

B. INTRODUCTION

Exposure of small mammals to a helium-based atmosphere has been shown to result in an increase in oxygen consumption. It has been assumed the increased metabolism is caused by the high thermal conductivity of helium and that, therefore, mammals need to produce sufficient additional heat to maintain body temperature in the face of increased convective heat loss (Leon and Cook, 1960). This effect may mask other factors which could also change the rate of oxygen consumption. Also, it is not clear whether acclimation or adaptation to a helium-based atmosphere occurs.

To investigate these questions, oxygen consumption studies in air and in helium-oxygen were conducted utilizing mice and rats chronically exposed in the CES-4 described in Section 2 of this report.

Previous studies on rats maintained in a 79:21 helium-oxygen environment at ambient pressure for two weeks indicated that oxygen consumption in helium remained elevated and that oxygen consumption in air was depressed in comparison with comparable air-maintained rats (Rhoades *et al.*, 1966). We had available for studies of the comparative acclimation of oxygen consumption rats maintained in helium-oxygen and nitrogen-oxygen for six months.

To our knowledge, comparative oxygen consumption studies of mice conceived,

born, and raised in helium-oxygen and never exposed to air, and mice born in air but maintained for a long period of time in helium-oxygen have not been made.

Since mammals increase heat production to compensate for increased heat loss in a helium atmosphere, oxygen consumption studies of a poikilotherm lacking an effective thermal regulation mechanism were conducted to uncover possible effects of helium on oxygen consumption that are not based on thermal effects. A logical choice for this study was the young boa constrictor. These animals are small enough to fit in the oxygen consumption chamber yet large enough to enable recording of measurable oxygen uptake.

In order to perform these oxygen consumption studies under constant environmental conditions, a constant temperature and relative humidity system, CES-5, was designed and built (cf. Figure 10).

C. METHODS

1. *Experimental plan*

Rats, parent generation mice, and F_2 generation mice were paired from Chambers A (helium chamber) and B (nitrogen chamber) for oxygen consumption studies conducted at ambient pressure and at about the same environmental temperature and relative humidity as the chronic exposure chambers (23 °C and 40–60% relative humidity), and at PCO_2 levels of less than 7 mm Hg.

Each animal served as its own control, and each experiment was designed to include a control segment which consisted of oxygen consumption measured in the gaseous environment in which the animal had been maintained; the experimental portion involved the other gaseous environment (e.g., helium-oxygen was the control for the helium chamber animals and air was the experimental atmosphere). About 10 minutes was allotted during each experiment for equilibration of CES-5, the oxygen consumption measuring system, and settling of the animals. Changing the gaseous environment was accomplished by flushing CES-5 *in toto*, utilizing its circulating pump.

The resultant data were statistically examined for differences in oxygen consumption in air and oxygen consumption in helium-oxygen between animals maintained and raised in these two gaseous environments.

2. *Equipment and materials*

A closed environment sytem, designated CES-5, was designed and built to permit volumetric oxygen consumption measurements of small animals at ambient pressure with rigidly controlled environmental temperature, carbon dioxide level, and relative humidity. It consisted of a water jacketed cylindrical plexiglass animal chamber, 40.5 cm long × 8.5 cm in diameter, with a volume of 9.2 liters; a water-jacketed gas heat exchanger; a Lauda K2/R circulating thermostatted water bath with a temperature range of 0–100 ± 0.05 °C, a Warren E. Collins one-liter recording spirometer with a bell factor of 4.56 ml/mm; a carbon dioxide absorbing scrubber apparatus which was immersed in a cooling bath to utilize the 'cold finger' principle for relative humidity

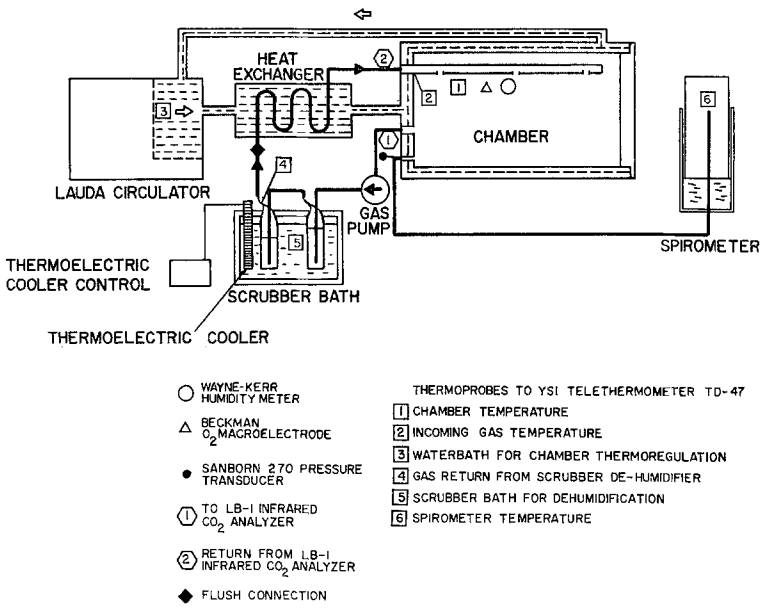

Fig. 10. Schematic representation of CES-5.

control; and a 9-liter/min diaphragm Dynapump for gas circulation (see Figure 10).

Oxygen consumption studies conducted with this apparatus utilized the principle that, in a steady state, the spirometer replaces the volume of oxygen consumed by the animals to maintain ambient pressure equilibrium. The decrease in volume in the spirometer is considered to be the oxygen consumption. Carbon dioxide was absorbed and maintained at constant, low, steady-state level by recirculation through the scrubber apparatus.

Provision was made to record experimental parameters on an eight-channel Beckman Offner Type R Oscillograph. The following system parameters were monitored and recorded continuously: carbon dioxide level (Beckman LB-1 Infrared Gas Analyzer with a recirculating flow rate of about 500 ml/min), temperatures throughout the system (YSI TD-47 Scanning Telethermometer), chamber pressure (Sanborn 270 Differential Pressure Transducer), spirometer excursion (W. E. Collins Recording One-Liter Spirometer), and oxygen partial pressure (Polarographic Coupler and a Beckman Macroelectrode, later modified by use of a Beckman 777 Electrode System with recorder output). Relative humidity of the chamber was monitored with a Wayne Kerr Humidity Meter.

CES-5 has a working volume of 13.2 liters which was calculated by dilution of carbon dioxide in the system with the spirometer closed and with slightly acid water in the scrubber jars. To accomplish this, 50 ml of air was removed from the chamber and an equal volume of 100% CO_2 was added. This was repeated four times, and the results were averaged. Analysis of CO_2 was done by the in-line infrared CO_2 analyzer.

Provision for changing the gaseous environment was made through a removable tubing connection between the scrubber apparatus and the gas heat exchanger. This was later modified by the addition of two 3-way valves in series. The location of this flush connection is shown in Figure 10.

The chamber temperature was maintained at 23.5 ± 0.5 °C and the relative humidity between 40 and 60%. Carbon dioxide was maintained below 1%, using 150 ml of 2N NaOH as the scrubber solution. The NaOH solution was changed daily.

The animals chosen for this study were two female rats each from the nitrogen chamber (designated N_2 rats) and from the helium chamber (designated He rats); three female parent generation mice from the nitrogen chamber (N_2P mice), and three from the helium chamber (HeP mice) (all of which were multiparous); and three nulliparous female F_2 generation mice from each of the chambers (HeF_2 mice and N_2F_2 mice). The animals of each set were studied as a group.

All the animals were restrained during the oxygen consumption experiments. The rats were individually confined in custom-made vinyl screen cages shaped to fit with close tolerance; the floor, front and back were plexiglass, the front having a porthole and the back being adjustable and having a tailhole. The rats were habituated to these cages before they were used in experiments. The mouse cage, made from copper wire mesh and plexiglass containers, consisted of four compartments; each individual partition measured 4 in. $\times 3\frac{1}{4}$ in. The mice would not tolerate body-sized cages. Restraints of this type were used to permit maximal gas exposure of the animals.

The gases used were room air, 80% helium – 20% oxygen, and >99% oxygen.

The system was calibrated and checked daily for leaks. Leak testing was accomplished by increasing and decreasing the pressure by 5–10 mm Hg, and monitoring its constancy with the pressure transducer.

For an experiment the animals were weighed, inserted into a restraining device, and placed in CES-5. The system was then sealed, flushed with the desired gases, and the spirometer flushed and filled with oxygen. Recording was started, and the system was allowed to equilibrate until relative humidity and CO_2 reached a stable level. Continuous recordings were made of spirometer excursion and CO_2 level.

The temperature measurement and recording cycled at 20-second intervals to each of the following locations: (1) the Lauda bath, (2) input gas to the chamber, (3) chamber gas, (4) spirometer, (5) scrubber bath, (6) gas returning from the scrubber, and (7) a calibration device set at 25 °C. Oxygen partial pressure was monitored continuously with a Beckman macroelectrode. Relative humidity was read at the beginning and end of each time segment of the oxygen consumption experiments and noted at the appropriate interval on the recording. Spirometer readings were also noted and entered on the recording. All experiments were conducted at ambient pressure, which was noted and recorded.

An experiment was divided into two sections, each of which consisted of four 10-min periods during exposure to one of the experimental atmospheres. The first 10-min period was allotted for stabilization (even spirometer tracing, stable CO_2 level, stable relative humidity, and settled animals), followed by the three 10-min oxygen consump-

tion measurement periods. The second section of the experiment consisted of a 10–12-min flush with the other gas atmosphere at a rate of about 5 liters/min, followed by the same four 10-min periods. During flushing, the spirometer stopcock was closed to the chamber, the CO_2 analyzer kept in operation, and the system closed except at the flush location. The animals remained in their restraints in the chamber.

The mice and rats were exposed to air for no longer than 10 min between removal from CES-4 and placement in the CES-5. They were returned to their respective chambers immediately after each experiment. None was accidentally or purposefully transferred from CES-4A (helium) to CES-4B (nitrogen).

Experiments of the following type were conducted:

(a) Oxygen consumption of rats was measured on four different days. On two of the days the first exposure was helium-oxygen followed by air; on the other two days the first exposure was air followed by helium-oxygen. The order was random. A total of four experiments on each pair of rats was performed.

(b) The oxygen consumption experiments on each set of mice (He P, N_2 P, He F_2, N_2 F_2) followed the same pattern. A total of six experiments on each set of mice were performed on six different days. On half of the days the experiments were ordered from air to helium-oxygen and on the other half from helium-oxygen to air; the ordering was random.

Oxygen consumption was calculated in ml of O_2/kg/min converted to STPD. The means and standard deviations were calculated and statistical comparisons made. The Fisher paired *t*-test for uncorrelated data of small samples (Guilford, 1965) was used to compare the oxygen consumption data of the various experiments.

During examination of the recordings of the experiments several were found to indicate malfunction of CES-5. These malfunctions were due to small leaks in the CES-5 system during the experiments, revealed by fluctuations in the P_{O_2} and pressure recordings. All experiments which showed these fluctuations were repeated, regardless of the values. As before, repeat experiments were done in pairs and in random order.

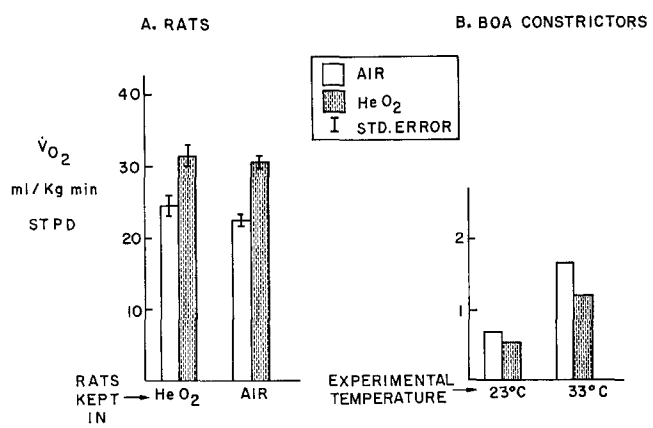

Fig. 11. Oxygen consumption of rats and boa constrictors. A: Comparison of response of air-raised (right) and helium-raised (left) rats with respect to their response to the HeO_2 atmosphere. B: Responses of boa constrictors to air and HeO_2 at two different temperatures. Boas were kept in air.

D. RESULTS

1. *Rats*

Oxygen consumption data from rat experiments are summarized in Table XII and presented graphically in Figure 11. A statistical comparison of the various situations is given in Table XIII.

TABLE XII

Oxygen consumption of rats in air and in helium-oxygen at 23° and 45–60% relative humidity

Order of Experiment		He rats O_2 ml/kg/min, STPD		N_2 rats O_2 ml/kg/min, STPD	
		In air	In HeO_2	In air	In HeO_2
Air to HeO_2		32.1	32.2	24.9	29.9
		29.3	32.4	23.2	31.6
		28.9	30.5	22.1	33.1
	$\bar{X}$	30.1	31.7	23.4	31.5
Air to HeO_2		21.0	38.0	19.5	32.5
		22.4	34.3	20.9	30.8
		20.6	34.3	20.7	32.3
	$\bar{X}$	21.3	35.5	20.3	31.9
HeO_2 to Air		21.4	26.5	25.3	29.2
		21.8	25.7	25.5	26.0
		21.0	23.0	26.4	26.9
	$\bar{X}$	21.4	25.1	25.7	27.4
HeO_2 to Air		25.9	33.1	20.7	30.0
		26.1	34.0	20.6	32.9
		24.9	33.4	20.6	31.4
	$\bar{X}$	25.6	33.5	20.6	31.4
± s.e.					
Overall mean		24.6 ± 1.4	31.5 ± 1.5	22.5 ± 0.8	30.6 ± 0.8
Weight in grams		306 and 307		337 and 251	

TABLE XIII

Statistical summary of oxygen consumption data, rats

Comparison	t	df	P	% Difference in V_{O2}
He rats vs. N_2 rats				
Air	1.593	11	>0.05	He rats 9% > N_2 rats
HeO_2	0.640	11	>0.05	He rats 3% > N_2 rats
Air vs. HeO_2				
He rats	4.114	11	<0.005	HeO_2 28% > air
N_2 rats	8.457	11	<0.001	HeO_2 36% > air
Order of exposure				
He rats in air	0.980	5	>0.10	–
He rats in HeO_2	1.761	5	>0.10	–
N_2 rats in air	0.916	5	>0.10	–
N_2 rats in HeO_2	1.398	5	>0.10	–

TABLE XIV

Oxygen consumption of mice and air and in HeO_2 at 23°C and 45–60% relative humidity

Order of experiment		HeP		N_2P		HeF_2		N_2F_2	
		Air	HeO_2	Air	HeO_2	Air	HeO_2	Air	HeO_2
Air to HeO_2		48.1	49.9	69.8	80.6	75.8	75.7	71.0	91.8
		41.5	58.4	71.8	77.4	66.6	80.2	67.3	84.7
		43.3	60.8	61.8	81.8	78.1	80.2	65.6	87.4
	$\bar{x}$	44.3	56.4	67.8	79.9	73.5	78.7	68.0	88.0
Air to HeO_2		55.4	85.4	47.7	58.0	38.4	51.6	47.9	65.0
		50.8	83.4	50.5	58.8	37.9	48.3	83.3	83.3
		62.2	87.6	48.2	60.9	41.3	50.2	48.3	81.2
	$\bar{x}$	56.1	85.5	48.7	59.2	39.2	50.0	50.3	76.5
Air to HeO_2		36.4	48.8	40.9	55.4	46.1	80.9	48.5	65.8
		39.8	57.0	43.4	51.9	42.8	82.4	65.3	66.0
		45.9	48.1	44.3	57.4	–	78.4	50.8	62.5
	$\bar{x}$	40.7	51.3	42.9	54.9	43.0	80.6	54.7	64.4
HeO_2 to Air		59.4	80.2	43.7	64.6	52.1	117.6	64.2	87.4
		65.4	72.9	41.1	65.7	56.5	113.9	43.9	97.6
		60.8	68.8	47.2	56.8	58.0	118.5	52.8	102.9
	$\bar{x}$	61.9	74.0	44.0	62.4	55.0	116.3	53.6	96.3
HeO_2 to Air		52.2	79.6	49.3	52.6	58.6	78.0	80.4	82.3
		55.8	75.4	45.2	57.9	56.5	83.0	83.1	88.4
		53.6	69.4	41.4	48.7	56.8	64.4	74.0	84.0
	$\bar{x}$	53.9	74.8	45.3	52.8	57.3	75.5	79.2	84.6
HeO_2 to Air		44.0	49.9	40.3	41.3	40.9	52.3	95.6	109.7
		47.0	55.4	41.9	47.7	52.6	66.2	59.6	108.8
		41.8	62.0	43.9	47.1	52.2	65.1	68.9	109.7
	$\bar{x}$	44.3	55.8	42.0	45.4	48.6	61.2	68.9	109.4
Overall mean		50.2	66.3	48.5	59.1	53.6	77.1	62.5	86.5
± s.e.		±2.0	±3.2	±2.3	±2.7	±2.8	±5.1	±3.4	±3.6
$\bar{x}$ weight in grams		48.5		48.5		34.7		32.3	

Figure 11A shows clearly that both pairs of rats responded in the same way to the HeO_2 environment. Statistically there was no difference in the response to either air or HeO_2 between the air-raised and helium-raised animals. Likewise, there was no difference in the magnitude of response to helium. A comparison of response with respect to order of presentation of the stimulus gas also showed no significant difference ($p > 0.10$). The increase in oxygen consumption due to the HeO_2 environment was, as expected, significant.

2. *Mice*

Data from the mouse experiments are given in Table XIV and Figure 12. The individual entries represent three successive 10-minute measurement periods. A number of these

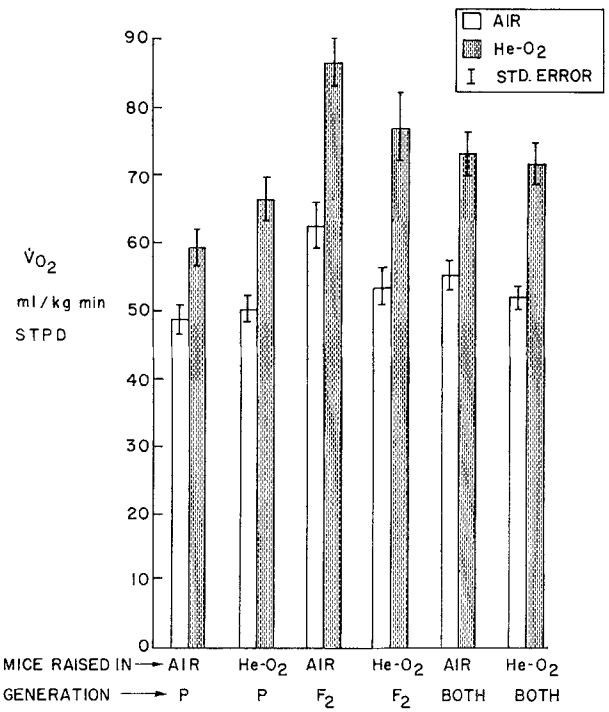

Fig. 12. Oxygen consumption of mice. The open bars show the mean oxygen consumption of mice in air, and the shaded bars the consumption of the same mice in HeO_2. Order of presentation of the gases was mixed, and had no effect.

categories were compared statistically using the paired *t*-test; statistical results and per cent differences are given in Table XV.

As was the case in the rat experiments, there was no difference in oxygen consumption in air or in HeO_2 due to the order of gas presentation. Each group of mice had a greater mean oxygen consumption in HeO_2 than in air, averaging 38% greater for the helium-raised mice and 31% greater for the air-raised mice. Since the purpose of the entire project was to investigate the degree of adaptation that animals show to a helium-oxygen atmosphere, we were interested in whether this difference in response (38% for the helium-raised animals as compared to 31% for the air-raised mice) represented a statistical difference. Increases in individual experiments were compared as ratios with the *t*-test and were found to have no statistical significance ($p > 0.7$ for the F_2 generation and > 0.2 for the parents.) This may be seen graphically in Figure 12, in that the relative increase due to HeO_2 seems to be about the same in all columns.

As might have been expected there were significant differences in oxygen consumption, both in air and in HeO_2, between the generations. Unfortunately, it was not possible to compare the two generations at the same time in their life cycle – the F_2 animals were much younger than the parents at the time of the measurements and would therefore have greater oxygen consumption on a weight basis even in the air environment.

TABLE XV

Statistical summary of oxygen consumption data, mice

Comparison	t	df	P	% Difference in V_{O_2}
He P vs. N_2 P				
Air	0.416	17	>0.05	–
HeO_2	1.726	17	>0.05	He P 12% > N_2 P
He F_2 vs. N_2 F_2				
Air	1.978	17	>0.05	N_2 F_2 16% > He F_2
HeO_2	1.501	17	>0.05	N_2 F_2 12% > He F_2
All He mice vs. all N_2 mice				
Air	1.253	35	>0.05	N_2 mice 7% > He mice
HeO_2	0.243	35	>0.05	
He P vs. He F_2				
Air	0.948	17	>0.05	He F_2 7% > He P
HeO_2	1.788	17	>0.05	He F_2 16% > He P
N_2 P vs. N_2 F_2				
Air	2.852	17	<0.02	N_2 F_2 29% > N_2 P
HeO_2	6.107	17	<0.001	N_2 F_2 46% > N_2 P
Air vs. HeO_2				
He P	4.282	17	<0.001	HeO_2 32%-air
N_2 P	2.372	17	<0.05	HeO_2 22% > air
He F_2	3.959	17	<0.001	HeO_2 44% > air
N_2 F_2	4.874	17	<0.001	HeO_2 38% > air
All He mice	5.585	35	<0.001	HeO_2 38% > air
All N_2 mice	4.373	35	<0.001	HeO_2 31% > air
Order of exposure				
He P in air	0.941	8	>0.10	–
He P in HeO_2	0.805	8	>0.10	–
N_2 P in air	0.466	8	>0.10	–
N_2 P in HeO_2	0.030	8	>0.10	–
He F_2 in air	1.000	8	>0.10	–
He F_2 in HeO_2	0.112	8	>0.10	–
N_2 F_2 in air	0.315	8	>0.10	–
N_2 F_2 in HeO_2	0.312	8	>0.10	–
All He mice in air	0.039	17	>0.10	–
All He mice in HeO_2	0.046	17	>0.10	–
All N_2 mice in air	0.198	17	>0.10	–
All N_2 mice in HeO_2	0.016	17	>0.10	–

E. BOA CONSTRICTORS

Since the bulk of evidence seems to support the hypothesis that the increased oxygen consumption in helium is due to its thermal properties, we thought it might be of interest to investigate an animal species that has a different type of thermal regulating

TABLE XVI

Oxygen consumption of boa constrictors in air and in helium-oxygen (averaged over 1-hour periods)

At 23 °C and 40–60 % relative humidity:

	O_2 ml/kg/min, STPD	
	In air	In HeO_2
	0.66	0.36
	0.73	0.73
$\bar{x}$	0.70	0.55

Weight, kg 1.64
Internal temperature 23.0 °C

At 33 °C and 40–60 % relative humidity:

	O_2 ml/kg/min, STPD	
	In air	In HeO_2
	1.63	1.12
	2.07	1.21
	1.85	1.21
	1.17	1.30
$\bar{x}$	1.68	1.21

Weight, kg 1.56 and 1.21
Internal temperature 33.0 °C and 32.8 ° C

system. The so-called 'cold-blooded' animals or heterotherms maintain a body temperature only slightly above that of the environment, and if cooled would be expected to lower rather than raise their metabolic rate.

The boa constrictor (*Constrictor constrictor*) was chosen for several reasons. Boas can be obtained in a suitable size range (large enough to have a measurable consumption yet small enough to fit the chamber), will rest quietly during the measurement, have an appropriate surface-to-weight ratio, and are easily handled and inexpensive.

The two boas weighed 1.2 and 1.5 kg and were each about 1.5 m in length. They were placed in the chamber in a mesh bag, allowed to equilibrate, and monitored for 1-hour periods in air. The chamber was then flushed and the measurements repeated with HeO_2. Experiments were conducted at 23 °C and were not replicated. One snake was measured at 23 °C for two 1-hour periods each in air and in He-O_2; two snakes were measured at 33 °C for four 1-hour periods each in air and in He-O_2. Results are shown in Table XVI and on Figure 11B.

F. DISCUSSION

1. *Rats and Mice*

The rats were successfully habituated to the restraining cages and presented no problem during the experiments. The data recorded were repeatable with little variation during an experiment or from day to day. The mice, on the other hand, were not ideal

experimental subjects for oxygen consumption studies, as their activity levels were highly variable and they did not become habituated to restraining cages within reasonable time limits.

Experimental order. The order of presentation of the experimental atmospheres apparently had no effect on the level of oxygen consumption; that is, statistically it made no difference in oxygen consumption if the helium or nitrogen rats and mice were exposed first to helium-oxygen, then to air, or first to air and then to helium-oxygen.

Oxygen consumption in air vs helium-oxygen. Both pairs of rats had increased oxygen consumption in helium-oxygen in comparison with air at a high level of confidence. The four sets of mice also had higher oxygen consumptions in helium-oxygen than in air. These results are in agreement with the findings of others (Leon and Cook, 1960; Rhoades *et al.*, 1966), and on mice raised in helium-oxygen and nitrogen-oxygen in this laboratory (Schreiner *et al.* 1965). This effect has been attributed, as previously mentioned, to the helium thermoconductivity-heat maintenance problem of mammals.

Chamber comparisons. Although all comparison groupings of He and N_2 mice and rats suggested that the He animals might have a higher mean oxygen consumption in air and in helium-oxygen than the comparable N_2 animals, none of these differences showed statistical significance. Thus, one cannot reliably conclude that the chronic exposure of mice and rats to helium alters their metabolic response to this gas.

The helium chamber rats, when compared to the nitrogen chamber rats, showed a trend toward higher oxygen consumptions in air, but these differences are not statistically significant. The data of Rhoades *et al.*, (1966) on the other hand, indicated that rats maintained in helium-oxygen for two weeks showed a depression of oxygen consumption when returned to air in comparison with air-maintained control rats. It should be noted that these experiments are not strictly comparable to those of Rhoades *et al.* Their deviations were found in the first few minutes after making the change, while in our case the animals were allowed to equilibrate to a steady state before we began to make measurements. Also, their animals were passed from He-O_2 to air (or the reverse) through 100% oxygen as an intermediate gas for 10 minutes. Our data do not support the view that rats become metabolically acclimated to helium during a 6-month exposure to this gas.

General comparisons. It was anticipated that the F_2 mice would have greater oxygen consumptions than the P mice strictly due to the well-established inverse relationship between age and oxygen consumption (Prosser and Brown, 1963; Richards, 1965). Our data support this relationship.

2. *Boa Constrictors*

Since only two experiments were performed, definitive statements would be premature. In both experiments, at 23 °C and at 33 °C, oxygen consumption was greater in air than in helium-oxygen. It is reasonable to suspect that a heterothermic animal would not have increased oxygen consumption in a helium-based atmosphere since it does not closely regulate its body heat. Cook (1950) found that lizards (*Cnemidophorus*

tessellatus and *Coleonyx variegatus*) produced less CO_2 in 80:20 helium-oxygen than in air; he did not measure oxygen consumption, and we did not measure CO_2 production.

3. *CES-5*

A critique of the CES-5 system is in order. The overwhelming problem associated with this principle of metabolism monitoring is that leaks are read on the spirometer tracing as oxygen consumed. Normally this can be corrected by maintaining a minimal pressure differential between the system and the atmosphere, and by not disturbing the system after it has once been sealed and leak tested. Some plumbing modifications can make this possible. Flushing the chamber to change the gases proved to be a minor problem. Initially, a removable tubing connection between the scrubber and heat exchanger was used (see Figure 10). Later, because of leakage and the difficulty encountered in rapidly reconnecting the tubing, the connection was modified by the addition of two 3-way valves in series.

Temperature control was excellent. During the experiments, chamber temperature varied less than 0.5 °C. Where small changes in oxygen are to be measured a thermostatted system is essential. Humidity control using the temperature of the scrubber is somewhat flow-limited, and at the flow rates used here the system sought a new steady state humidity level for different animal loads. Relative humidity was maintained within 8% of the desired point with two rats or three mice in the chamber. This was no problem in these experiments, but in cases where precise humidity control is necessary the flow will have to be increased or the scrubber temperature more appropriately matched to the load.

A liquid carbon dioxide scrubber, in addition to its application as a source of humidity control, has merit for another reason. Aliquot samples of the scrubber solution can be periodically extracted and analyzed, allowing the computation of carbon dioxide production. After the equilibration portion of the experiment, the scrubber maintained a steady state carbon dioxide level at less than 1 %.

References

Allen, L. C.: 1965, 'The Formation of Noble Gas Compounds', *Ann. N.Y. Acad. Sci.* **118**, 883–98.

Bennett, P. B.: 1966, *The Aetiology of Compressed Air Intoxication and Inert Gas Narcosis*, Pergamon Press, London.

Bergmeyer, H. V. and Bernt, E.: 1963, 'Malic Dehydrogenase', in *Methods of Enzymatic Analysis* (ed. by H. V. Bergmeyer), Academic Press, New York.

Bonura, M. S. and Nelson, W. G., *et al.*: 1967, Engineering Criteria for Spacecraft Cabin Atmosphere Selection, NASA CR-891.

Brooksby, G. A., Dennis, R. L., and Staley, R. W.: 1966, 'Effect of Continuous Exposure of Rats to 100% Oxygen at 450 mm Hg for 64 Days', *Aerospace Med.* **37**, 243–46.

Bruemmer, J. H., Brunetti, B. B., and Schreiner, H. R.: 1967, 'Effects of Helium Group Gases and Nitrous Oxide on HeLa Cells', *J. Cell. Physiol.* **69**, 385–92.

Bücher, T. and Klingenberg, M.: 1958, *Angew. Chem.* **70**, 552.

Consolazio, C. F., Johnson, R. E., and Pecora, L. J.: 1963, *Physiological Measurements of Metabolic Function in Man*, McGraw-Hill, New York.

Cook, S. F.: 1950, 'The Effect of Helium and Argon on Metabolism and Metamorphosis', *J. Cellular Comp. Physiol.* **36**, 115–27.

Cook, S. F. and Leon, H. A.: 1959, Physiological Effects of Inert Gases, AFMDC TR-59-26, Holloman AFB, Air Force Missile Development Center, New Mexico, U.S.A.

Cook, S. F., South F. E., and Young, D. R.: 1951, 'Effect of Helium on Gas Exchange in Mice', *Am. J. Physiol.* **164**, 248–50.

Delbrück, A., Zebe, E., and Bücher, T.: 1959, 'The Activity of Enzymes of the Energy-Producing Metabolism in Flight Muscle, Jumping Muscle and Fat Body of *Locusta migratoria*, and Their Cytological Distribution', *Biochem. Z.* **331**, 273–96.

Dixon, M. and Webb, E. C.: 1964, *Enzymes*, Academic Press, New York.

Epperson, W. L., Quigley, D. G., Robertson, W. G., Behar, V. S., and Welch, B. E.: 1966, 'Observations on Man in an Oxygen-Helium Environment at 380 mm Hg Total Pressure. III. Heat Exchange', *Aerospace Med.* **37**, 457–62.

Estabrook, R. W.: 1965, 'Discussion', in *Control of Energy Metabolism* (ed. by B. Chance, R. W. Estabrook, and J. R. Williamson), Academic Press, New York.

Featherstone, R. M. and Muehlbaecher, C. A.: 1963, 'The Current Role of Inert Gases in the Search for Anesthesia Mechanisms', *Pharmacol. Rev.* **15**, 97–121.

Frenkel, R.: 1965, 'Enzyme Profile of Beef Heart Supernatant Fraction', in *Control of Energy Metabolism* (ed. by B. Chance, R. W. Estabrook, and J. R. Williamson), Academic Press, New York.

Guilford, J. P.: 1965, *Fundamental Statistics in Psychology and Education*, McGraw-Hill, New York.

Glock, G. E. and McLean, P.: 1954, 'Levels of Enzymes of the Direct Oxidative Pathway of Carbonhydrate Metabolism in Mammalian Tissues and Tumors', *Biochem. J.* **56**, 171–75.

Green, D. E.: 1936, 'The Malic Dehydrogenase of Animal Tissues', *Biochem. J.* **30**, 2095–2110.

Hamilton, R. W., Jr., Doebbler, G. F., Nuermberger, C. H., and Schreiner, H. R.: 1966a, 'Biological Effects of Space Cabin Atmospheres: Exposure of Rabbits to Helium, Neon, Nitrogen, Argon and Oxygen', *Physiologist* **9**, 197.

Hamilton, R. W., Jr., Doebbler, G. F., Nuermberger, C. H., and Schreiner, H. R.: 1966b, Biological Evaluation of Various Space Cabin Atmospheres, Final Report to USAF School of Aerospace Medicine, Contract AF41(609)-2711, Union Carbide Corporation, Tonawanda, N.Y.

Hamilton, R. W., Jr., MacInnis, B. J., Trovato, L. A., and Schreiner, H. R.: 1966c, 'Biological Effects of Helium on Man: Results of a Multi-Day Exposure to This Gas at 20 Atmospheres. II. Physiological Evaluation', *Aerospace Med.* **37**, 281–82.

Hamilton, R. W., Jr. and Schreiner, H. R.: 1967, 'Effect of Helium, Neon, Nitrogen, and Argon on the Relative Susceptibility of Animals to Altitude Decompression Sickness', presented at the 38th Annual Meeting of the Aerospace Medical Association, Washington, D.C. April 10–13, 1967.

Hamilton, R. W., Jr., Cohen, J. D., Doebbler, G. F., Exposito, L. F., King, J. M., and Schreiner, H. R.: 1969, Biochemical and Metabolic Effects of a Six-Month Exposure of Small Animals to a Helium-Oxygen Atmosphere, NASA CR-1372.

Hargreaves, J. J., Robertson, W. G., Ulvedal, F., Zeft, H. J., and Welch, B. E.: 1966, 'Study of Man During a 56-Day Exposure to an Oxygen-Helium Atmosphere at 258 mm Total Pressure. I. Introduction and General Experimental Design', *Aerospace Med.* **37**, 552–55.

Hartshorne, D. J. and Perry, S. V.: 1962, 'A Chromatographic and Electrophoretic Study of Sarcoplasm From Adult- and Foetal-Rabbit Muscles', *Biochem. J.* **85**, 171–77.

Helvey, W. M., Albright, G. A., Benjamin, F. B., Gall, L. S., Peters, J. M., and Rind, H.: 1965, 'Effects of Prolonged Exposure to Pure Oxygen on Human Performance', in *Gaseous Environmental Considerations and Evaluation Programs Leading to Spacecraft Atmosphere Selection* (ed. by E. L. Michel, G. B. Smith, and R. S. Johnston), TND-2506, NASA, Washington, D. C.

Hess, B. and Brand, K.: 1965, 'Enzyme and Metabolic Profiles', in *Control of Energy Metabolism* (ed. by B. Chance, R. W. Estabrook, and J. R. Williamson), Academic Press, New York.

Higgins, J.: 1965, 'Dynamics and Control in Cellular Reactions', in *Control of Energy Metabolism* (ed. by B. Chance, R. W. Estabrook, and J. R. Williamson), Academic Press, New York.

Kissane, J. Q.: 1967, Study of the Effects of a Helium-Oxygen Atmosphere on Nucleotide Cofactors of Cellular Oxidative Systems, Report No. IITI-L6045-1 to the Natl. Aeron. Space Admin., IIT Research Institute, Chicago.

Lauryssens, M. G. and Lauryssens, M. J.: 1964, 'Electrophoretic Distribution Pattern of Lactate Dehydrogenase in Mouse and Human Muscular Dystrophy', *Clin. Chim. Acta* **9**, 273–76.

Leon, H. A. and Cook, S. F.: 1960, 'A Mechanism by Which Helium Increases Metabolism in Small Mammals', *Am. J. Physiol.* **199**, 243–45.

Lillie, R. D.: 1954, *Histopathic Technic and Practical Histochemistry*, McGraw-Hill, New York.

Löhr, G. W. and Waller, H. D.: 1963, Glucose-6-Phosphate Dehydrogenase in *Methods of Enzymatic Analysis* (ed. by H. V. Bergmeyer), Academic Press, New York.

Maio, D. A. and Neville, J. R.: 1966, The Effect of Chemically Inert Gases on Oxygen Consumption in Living Tissues, SAM-TR-66-109, Brooks AFB, USAF School of Aerospace Medicine, Texas, U.S.A.

Meister, A.: 1950, 'Lactic Dehydrogenase Activity of Certain Tumors and Normal Tissues', *J. Natl. Cancer Inst.* **10**, 1263–71.

Prosser, C. L. and Brown, Jr., F. A.: 1963, *Comparative Animal Physiology*, W. B. Saunders Co., Philadelphia.

Rendon, L.: 1965, Electrophoretic Separations on Acrylamide Gels: Disc. Electrophoresis, AMRL-TR-65-202, Wright-Patterson AFB, Aerospace Med. Res. Lab., Ohio.

Rhoades, R. A., Wright, R. A., and Weiss, H. S.: 1966, Metabolic Depression in Animals Exposed to Air After Living in a Helium-Oxygen Environment', *Proc. Soc. Explt. Biol. Med.* **124**, 176–80.

Richards, D. W.: 1965, 'Pulmonary Changes Due to Aging', in *Handbook of Physiology. II. Respiration* (ed. by W. O. Fenn and H. Rahn), Am. Physiol. Society, Washington.

Rinfret, A. P. and Doebbler, G. F.: 1961, 'Physiological and Biochemical Effects and Applications', in *Argon, Helium and the Rare Gases*, (ed. by G. A. Cook), Interscience, New York.

Rodgers, S. H.: 1966, The Oxygen Consumption of Rat Tissues in the Presence of Nitrogen, Helium or Hydrogen, Ph.D. Thesis, The University of Rochester, New York.

Roth, E. M.: 1964, Space Cabin Atmospheres. II. Fire and Blast Hazards, Report SP-48, NASA, Washington, D. C.

Roth, E. M.: 1965, Space Cabin Atmospheres. III. Physiological Factors of Inert Gases, Report SP-117, NASA, Washington, D.C.

Schmidt, E. and Schmidt, F. W.: 1960, 'Enzyme Patterns of Human Tissues', *Klin. Wschr.* 957–62.

Schreiner, H. R.: 1964, The Physiological Effects of Argon, Helium and the Rare Gases, Technical Report I to the Office of Naval Research. Contract Nonrr-4115(00), Union Carbide Corp., Tonawanda, New York.

Schreiner, H. R.: 1966, 'Interaction of Inert Gases With Molecular Cell Processes', *Biometerol.* **2**, 339–49.

Schreiner, H. R., Bruemmer, J. H., and Doebbler, G. F.: 1965, The Physiological Effects of Argon, Helium and the Rare Gases, Technical Report II to the Office of Naval Research, Contract Nonr-4115(00), Union Carbide Corp., Tonawanda, New York.

Schreiner, H. R., Gregoire, R. C., and Lawrie, J. A.: 1962, 'New Biological Effect of the Gases of the Helium Group', *Science* **136**, 653–54.

South, F. E. and Cook, S. F.: 1953, 'Effect of Helium on the Respiration and Glycolysis of Mouse Liver Slices', *J. Gen. Physiol.* **36**, 513–28.

Stengel, A. and Fox, H.: 1915, *A Textbook of Pathology*, Saunders, Philadelphia.

Swisher, S. N. and Fisher, C. L.: 1966, 'Hematological Changes in Space Flight', *Physiologist* **9**, 328.

Tepperman, J. and Tepperman, H. M.: 1965, 'On the "Biochemical Imprinting" of Metabolic Experience in Liver Cells', in *Control of Energy Metabolism* (ed. by B. Chance, R. W. Estabrook, and J. R. Williamson), Academic Press, New York.

Weiss, H. S. and Rhoades, R. A.: 1965, Biological Effects of Prolonged Exposure of Small Animals to Unusual Gaseous Environments, Report 1492-5, NASA, Ohio State Univ. Res. Fdn., Columbus.

Wroblewski, F.: 1957, 'The Clinical Significance of Lactic Dehydrogenase Activity in the Milieu Interieur', *Scand. J. Clin. Lab. Invest.* **10**, 230–53.