NASA CONTRACTOR REPORT

NASA CR-1372

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

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Prepared by
UNION CARBIDE CORPORATION
Tonawanda, N. Y.
for Ames Research Center

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION • WASHINGTON, D. C. • OCTOBER 1969

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Prepared under Contract No. NAS 2-3900 for
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NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

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I. ABSTRACT

Helium has been suggested as the inert gas diluent to be used in a spacecraft cabin atmosphere. While it may offer some advantages in case decompression is required, this gas has an established accelerating effect on metabolism in mammals, and the long-term biochemical adaptations to this increase have been an unknown factor. In this experiment rats and mice were exposed for periods of up to six months at 24°C, and two successive generations of mice were raised in a ground-level chamber system filled with 80 per cent helium - 20 per cent oxygen. 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.

II. 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 b). 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 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 (Bennett, 1966) and general anesthesia by xenon. These effects are attributed to weak intermolecular forces; they are not expected to be seen with helium (Schreiner, 1963). 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, Gregoire, and Lawrie, 1962) and mammalian cell systems (Bruemmer, Brunetti, and Schreiner, 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., 1966 b) 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 portentious 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.

III. 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 produce 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 present 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 the oxygen consumption was seen in mice breathing an 80:20 mixture of helium and oxygen (Cook, South, and Young, 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, Bruemmer, and Doebbler, 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. (1966 a) 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 per cent helium - 21 per cent 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 per cent helium - 21 per cent 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, Bruemmer, and Doebbler, 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 measurements 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) reduces 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., 1966 c).

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 per cent 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 Section V of this report, contains two completely separate but identical chambers identified as Chamber A and Chamber B (cf.

Table 1 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.

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 169 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 from 0.1 to 0.6 per cent and nitrogen levels, particularly after the initial shakedown of the system, rarely exceeded 1 per cent with an average value of about 0.5 per cent. Relative humidity was maintained between 40 and 60 per cent and an automatic light-dark cycle of 12 hours was maintained throughout the experiment (lights on 7 AM - 7 PM, lights off 7 PM - 7 AM) to achieve standard diurnal conditions.

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 per cent helium - 80 per cent oxygen.
Chamber B	Semi-closed environmental system containing 20 per cent oxygen - 80 per cent 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.

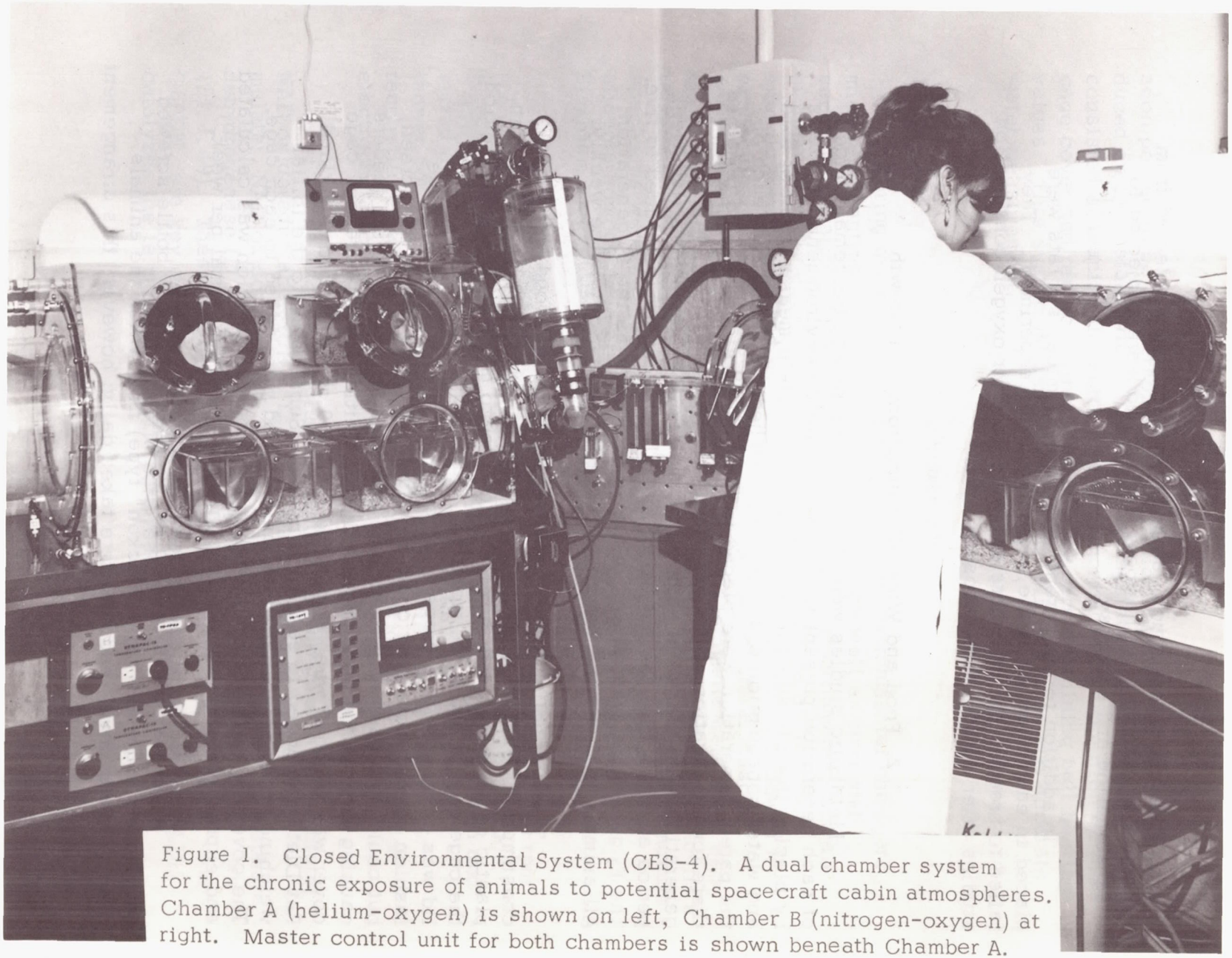

Figure 1. Closed Environmental System (CES-4). A dual chamber system for the chronic exposure of animals to potential spacecraft cabin atmospheres. Chamber A (helium-oxygen) is shown on left, Chamber B (nitrogen-oxygen) at right. Master control unit for both chambers is shown beneath Chamber A.

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, 11-1/2 in. x 6 in. x 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 and attached in a hinge fashion so that more food could be added easily by lifting the screen, but 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 biochemical analyses. Liver specimens were fixed and stained for glycogen (semi-quantitative) using the periodic acid-Schiff's technique (cf. Section III. 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 per cent (w/v) homogenate.

Homogenates were centrifuged in a No. 40 rotor at 20,000 rpm (26 - 36,000 x 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 - lactate + NAD = pyruvate + $NADH_2$;

MDH catalyzes the citric acid cycle reaction:

L - malate + NAD = oxaloacetate + $NADH_2$;

G6PDH catalyzes the hexose monophosphate shunt reaction:

D - glucose-6-P + NADP = 6-phosphogluconate + $NADPH_2$.

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} μ moles 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 was effected on cellulose acetate strips (12 x 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 below:

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 per cent Ponceau S in 5 per cent TCA, 5 min.	Amido Black, 60 min.
Destaining	Three times, 5 per cent acetic acid	Electrophoretic destaining in 7 per cent acetic acid. 15 ma/tube, 90 min.
Detection	Photovolt Densitometer wet, uncleared strips, 505 nm filter, response setting 5	Photographic, high contrast film.

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 II). 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 have resulted in disturbances of the red blood cell system in chamber tests (Helvey et al., 1965; Brooksby, Dennis, and Stanley, 1966) and spaceflights (Swisher and Fischer, 1966). It has been hypothesized that these hematologic effects were a result, at least in part, of increased levels of oxygen partial pressure.

Table II

Supernatant Fraction	Protein Concentration mg/ml [4]	Liver Homogenate Enzyme Activities, units/ml [5]		
		MDH	G6PDH	LDH
Crude[1]	24.5	1.65×10^5	2.73×10^3	3.96×10^4
Mitochondria-free[2]	9.2	1.53×10^5	3.10×10^3	3.92×10^4
True[3]	5.2	1.37×10^5	3.64×10^3	3.84×10^4

[1]Supernatant following centrifugation (600 x g, 10 min.).

[2]Supernatant following centrifugation (8500 x g, 10 min.).

[3]Supernatant following centrifugation (18000 x g, 60 min.).

[4]Determined by absorbance at 280 mμ relative to albumin.

[5]Homogenate: 10 per cent wet weight tissue in distilled water, prepared with Potter-Elvehjem homogenizer at 0°C.

Figure 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.

Hamilton et al. (1966 b) 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 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 determined 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

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*Ortho Pharmaceutical Corporation, Raritan, New Jersey.

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 III. 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 III.

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

Table III

STATISTICAL SUMMARY

Food and Water Consumption of Mice Maintained in Chambers A and B

Comparison	t	df	P	Result
Food Consumption: gm/gm mouse/wk				
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

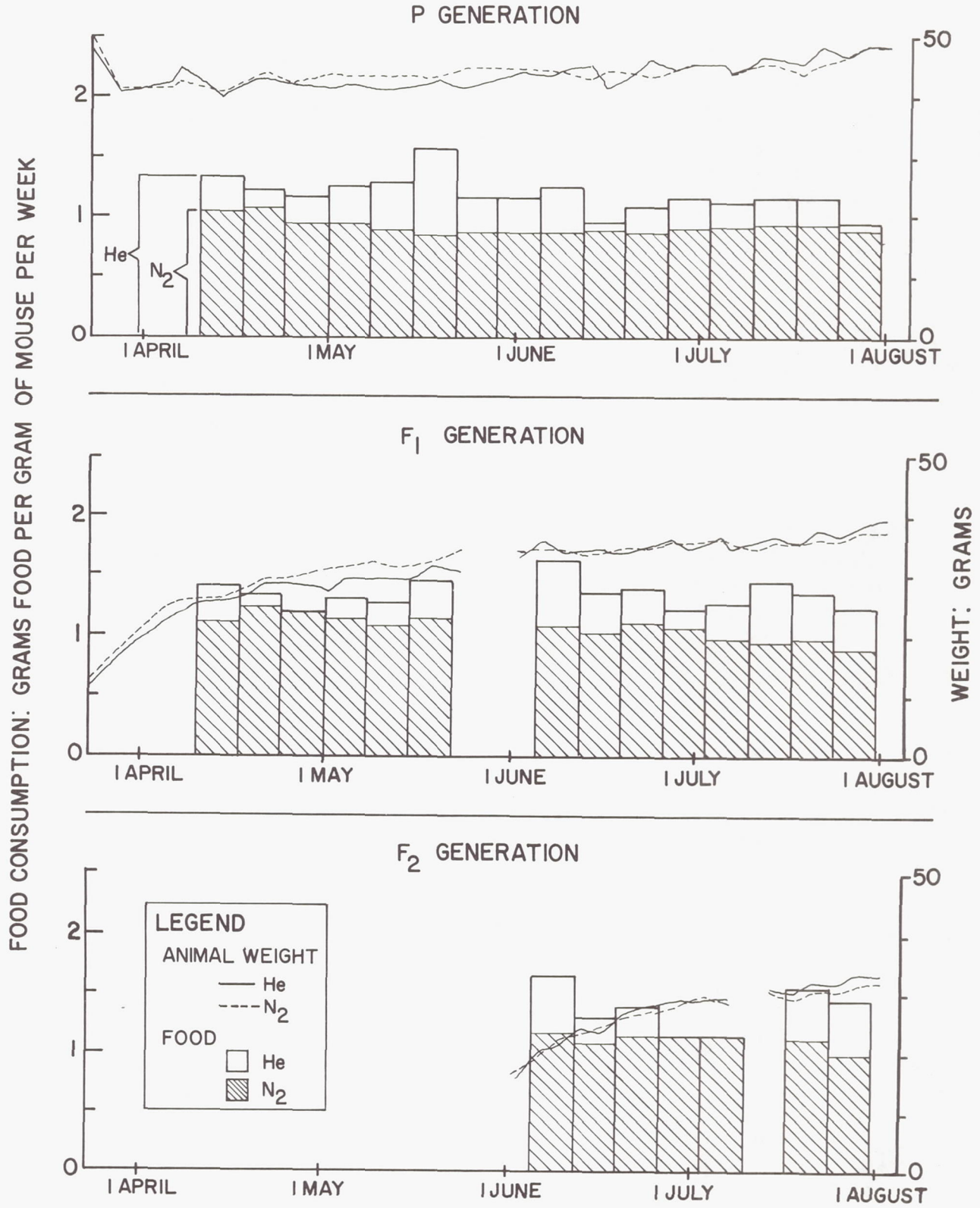


Figure 3. Food Consumption and Growth of Mice Chronically Exposed to Helium-Oxygen and Nitrogen-Oxygen at Ground Level Pressure.

Generation	P		F_1		F_2	
Inert Gas	He	N_2	He	N_2	He	N_2
Food Consumption (g/g mouse/wk.)	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.)

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 III). Mean values of water consumption ± one standard deviation and number of measurements (in parentheses) are given below for animal populations of five mice per cage. A statistical summary is given in Table III.

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

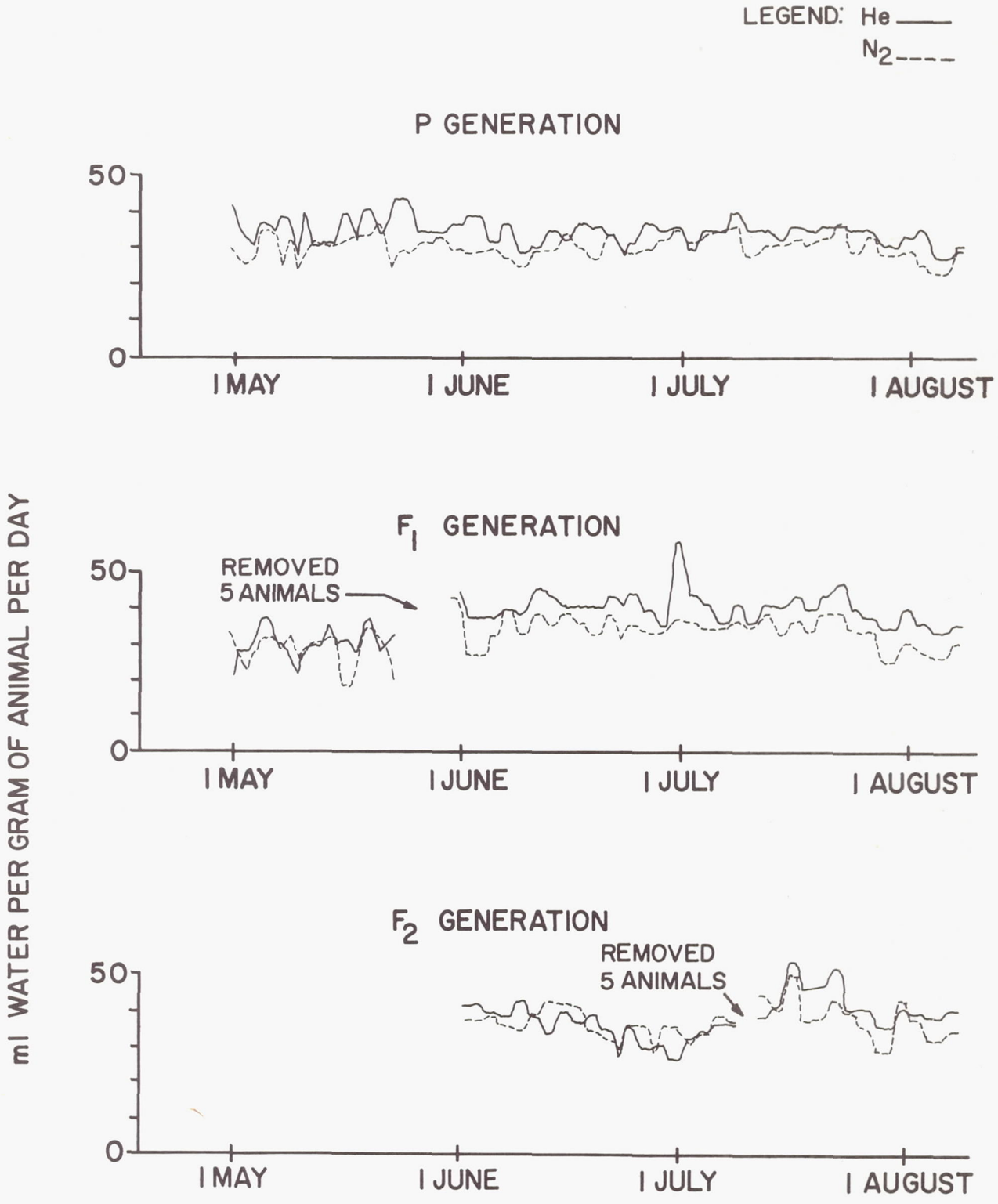


Figure 4. Water Consumption of Mice Chronically Exposed to Helium-Oxygen and Nitrogen-Oxygen at Ground Level Pressure.

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 per cent 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 population (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 26,000 x 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 IV 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 per cent of the mean; in several instances (i.e. some LDH and most G6PDH) it was as great as 50 per cent 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 (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) ($P > 0.20$). In no other comparison of groups were differences found significant at $P \leq 0.05$.

Table IV

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)

Footnotes:

Values are averages ± 1 standard deviation.
Numbers in parentheses indicate numbers of individual animals used.
LDH = lactate dehydrogenase [(units/GM fresh tissue) x 10^{-5}].
MDH = malate dehydrogenase [(units/GM fresh tissue) x 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 $F_1 + F_2$.

Table V

Relative Dehydrogenase Composition of Different Mouse Tissues

Enzyme	Tissue			Reference
	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 Dehydrogenase	100	37	11	Glock and McLean (1954)*
	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.

From the pooled group data of Table IV, 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 Bucher and Klingenberg (1958) and Delbruch, Zebe, and Bucher (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 distributions. 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 >>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 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 V 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) (c. f. 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) 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 per cent of the total protein; bands two and ten each contributed about 15 per cent 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 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

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 VI. 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. Hemogrlobin values for helium mice of all three generations did not differ significantly from control values obtained from nitrogen animals.

4. Pathology

The detailed results of histopathological examinations of kidney samples are presented in Table I of the Appendix to this report. A few round cell foci were noted but 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.

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 per cent of the total protein; bands Nos. 5, 6, and 7 constituted each about 10 per cent of the total. The electrophoretic patterns of the F_2 group livers showed in general a decrease of up to 50 per cent 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 per cent 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 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 μg 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 ± 1 SD)

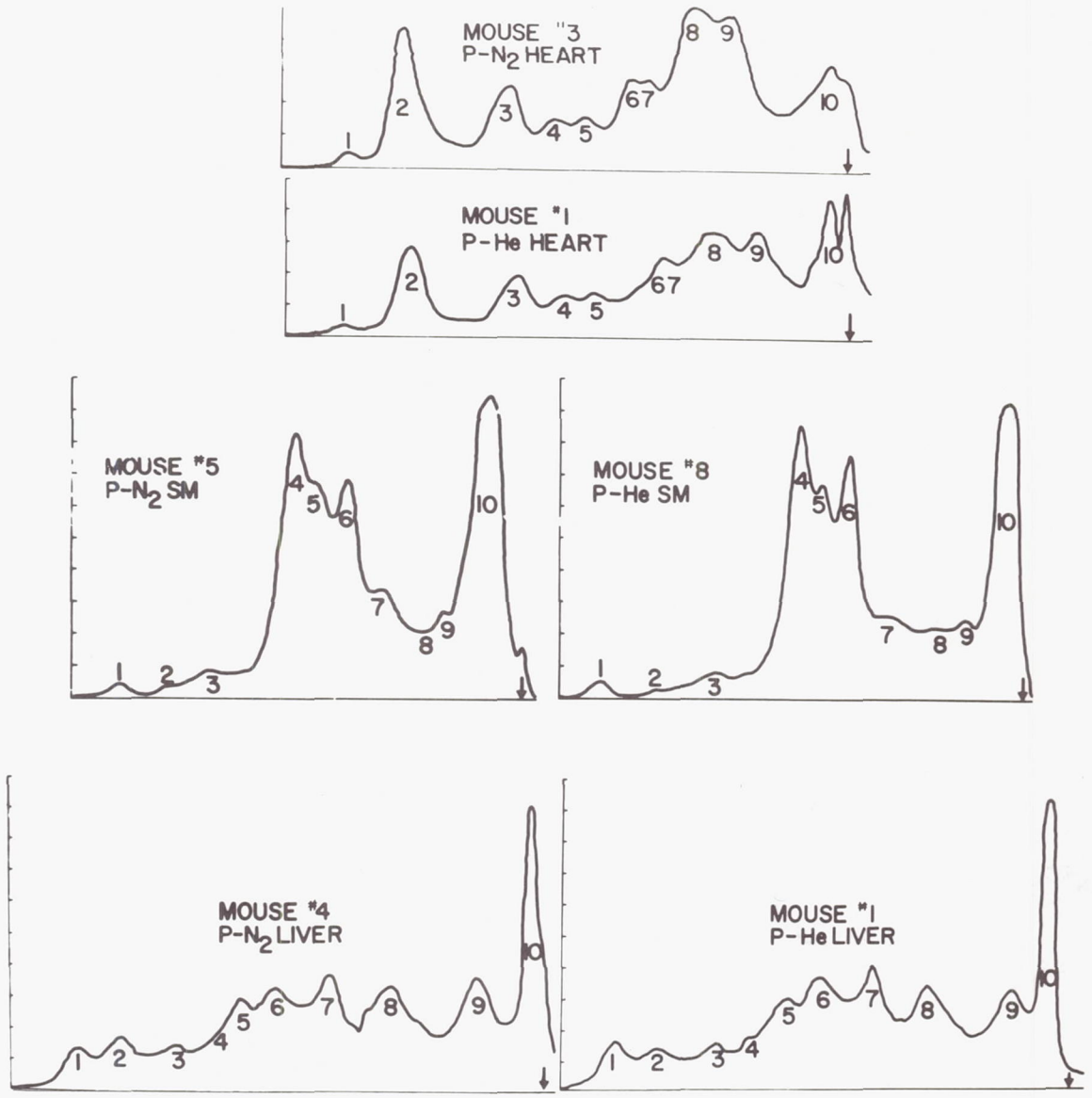


Figure 5. Representative Cellulose Acetate Strip Electrophoresis Patterns of Proteins of Mouse Tissue Extracts. Patterns are densitometer traces (arbitrary intensity scale) of actual strips. "P" refers to parent (initial) population; He and N_2 refer to animals from the helium-oxygen or nitrogen-oxygen environments respectively. Tissues are identified by name or for skeletal muscle (SM).

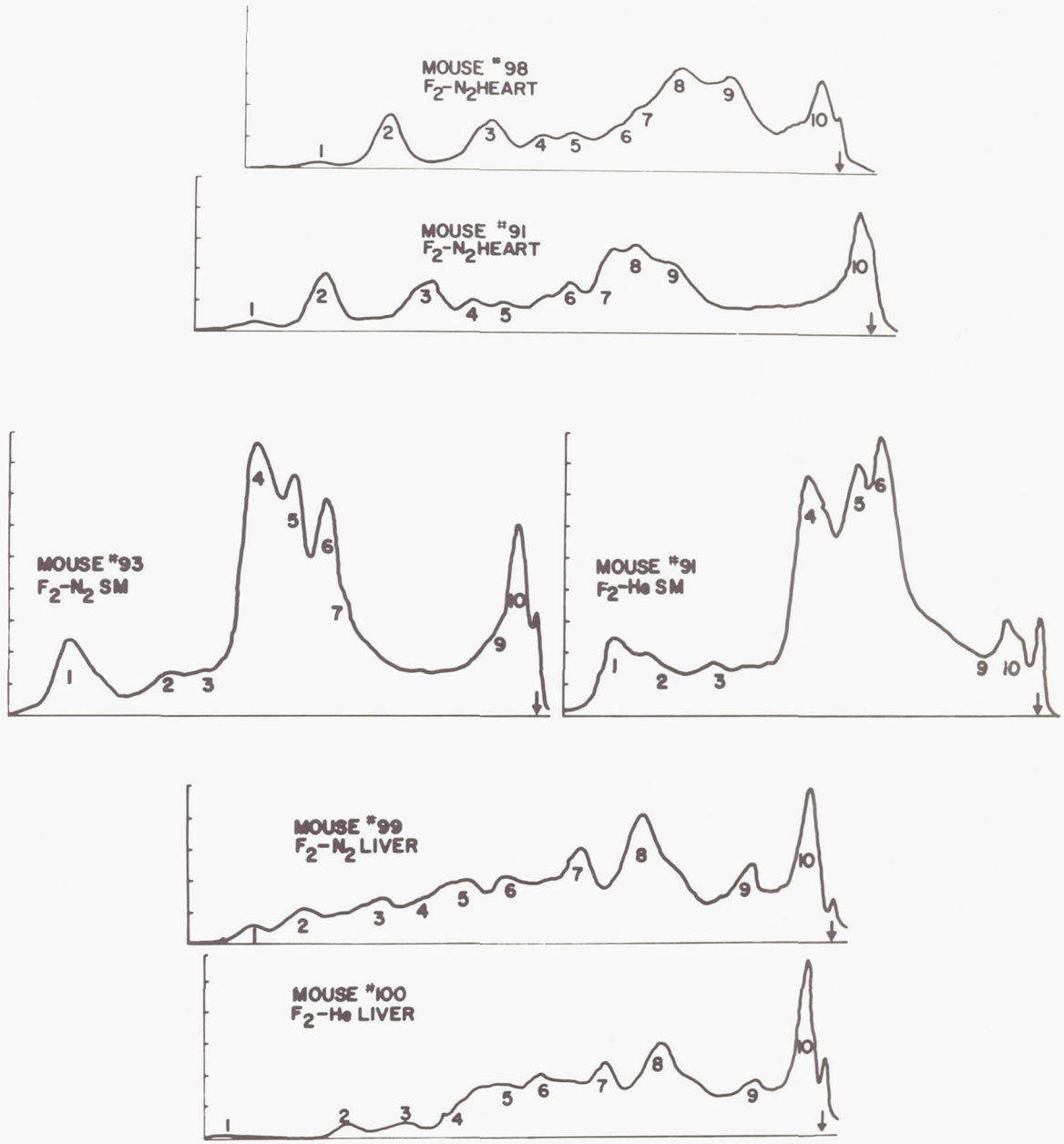


Figure 6. Representative cellulose acetate strip electrophoresis patterns of proteins of mouse tissue extracts. Symbols and conditions are as in Figure 5. F_2 refers to second generation animals born in the chamber environments.

Figure 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.

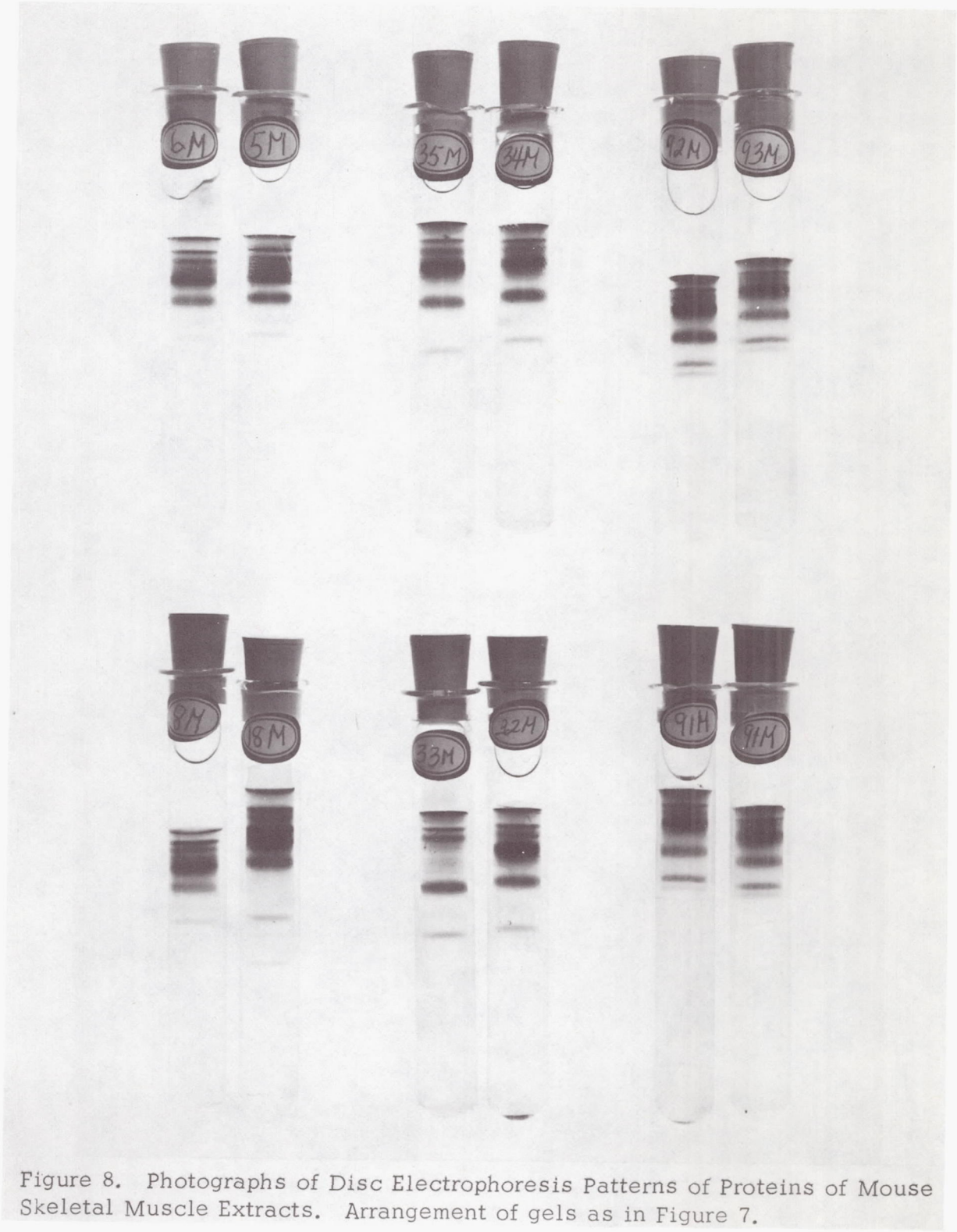


Figure 8. Photographs of Disc Electrophoresis Patterns of Proteins of Mouse Skeletal Muscle Extracts. Arrangement of gels as in Figure 7.

Figure 9. Photographs of Disc Electrophoresis Patterns of Proteins of Mouse Liver Extracts. Arrangement of gels as in Figure 7.

TABLE VI

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

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 VII. 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.

Mean organ weights of female mice randomly selected from the P, F_1 and F_2 generations are shown in Table VIII. 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 IV 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 to our expectations. The important corrolary 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 commeasurate increase noted in the food consumption of these animals). The less crowded cage possibly had a lower relative humidity.

TABLE VII

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	++++

TABLE VIII

ORGAN WEIGHTS OF FEMALE MICE CHRONICALLY EXPOSED TO HELIUM-OXYGEN AND NITROGEN-OXYGEN AT GROUND LEVEL PRESSURE

	Liver		Kidney		Heart		Diaphragm Wet		Diaphragm Dry	
					P Generation					
	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 per cent of whole body weight

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 exzymes 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. Harshorne 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 III, 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), electrophoretic (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 pattern 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 defintely 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 pattern 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 (cf. Appendix, Table II). 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 differentiallly.

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.

IV. 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 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 chambers described in Section III 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 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. Figures 10 and 11).

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 system, 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 control; and a 9-liter/min. diaphragm Dynapump for gas circulation (see Figures 10 and 11).

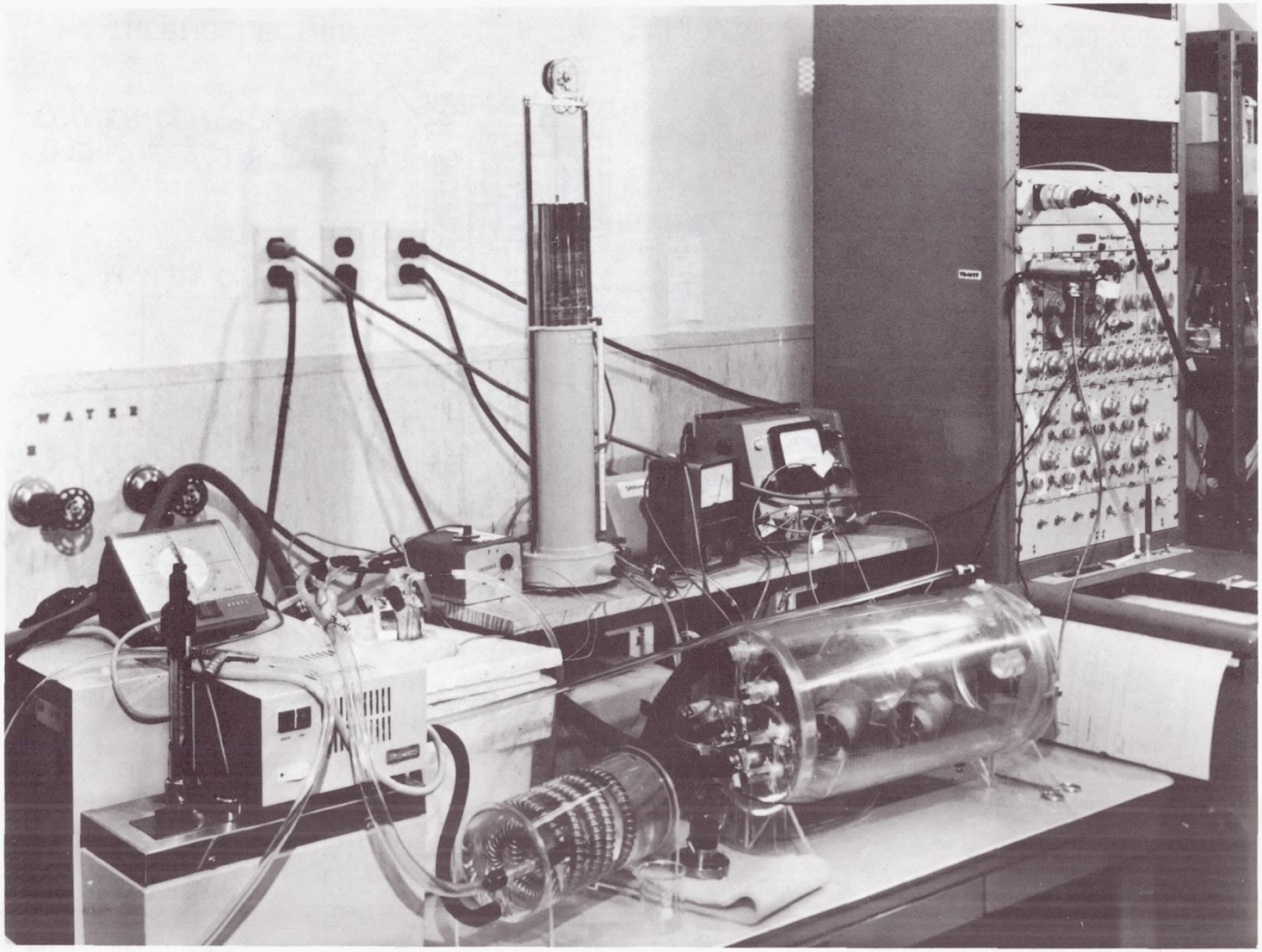


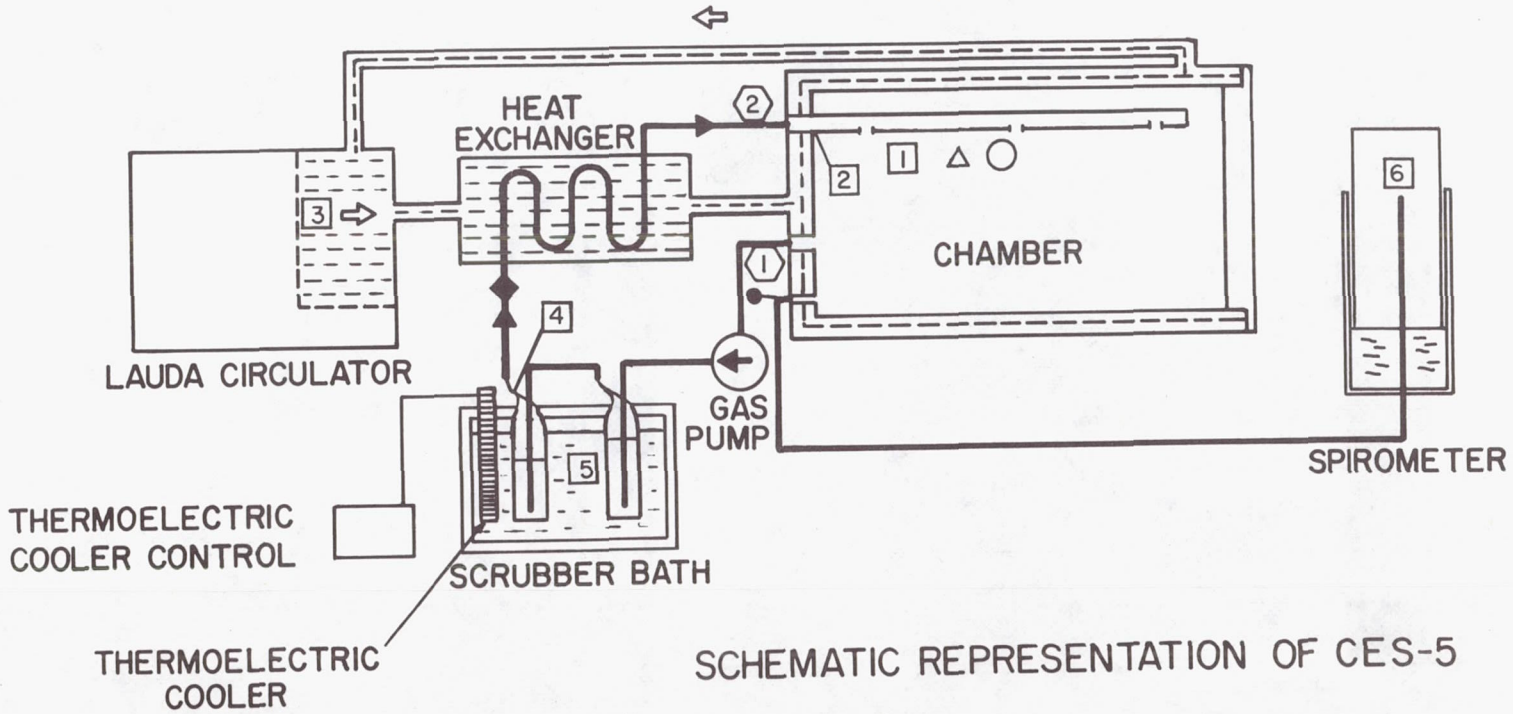
Figure 10. CES-5 in Operation. In the foreground, shown from left to right, are: the Lauda circulating water bath, gas heat exchanger, pressure transducer, animal chamber with two rats in restraining cages, and Offner recorder. In the background (from right to left) are the YSI cycling Telethermometer, humidity meter, spirometer, and the scrubber bath with its immersion cover. The carbon dioxide analyzer is on one of the shelves to the right, off camera.

LEGEND:

- ○ WAYNE-KERR HUMIDITY METER
- △ BECKMAN O_2 MACROELECTRODE
- ● SANBORN 270 PRESSURE TRANSDUCER
- ⬡1 TO LB-I INFRARED CO_2 ANALYZER
- ⬡2 RETURN FROM LB-I INFRARED CO_2 ANALYZER
- ◆ FLUSH CONNECTION

THERMOPROBES TO YSI TELETHERMOMETER TD-47

- [1] CHAMBER TEMPERATURE
- [2] INCOMING GAS TEMPERATURE
- [3] WATERBATH FOR CHAMBER THERMOREGULATION
- [4] GAS RETURN FROM SCRUBBER DE-HUMIDIFIER
- [5] SCRUBBER BATH FOR DEHUMIDIFICATION
- [6] SPIROMETER TEMPERATURE



SCHEMATIC REPRESENTATION OF CES-5

Oxygen consumption studies conducted with this apparatus utilized the principle that, in a steady state, the spriometer 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 record 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 11.

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_2 P mice), and three from the helium chamber (He P mice) (all of which were multiparous); and three nulliparous female F_2 generation mice from each of the chambers (He F_2 mice and N_2 F_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 (Figure 12); 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. x 3-1/4 in. (Figure 12). 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 consumption measurement periods. The second section of the experiment consisted of a 10-12-minute 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.

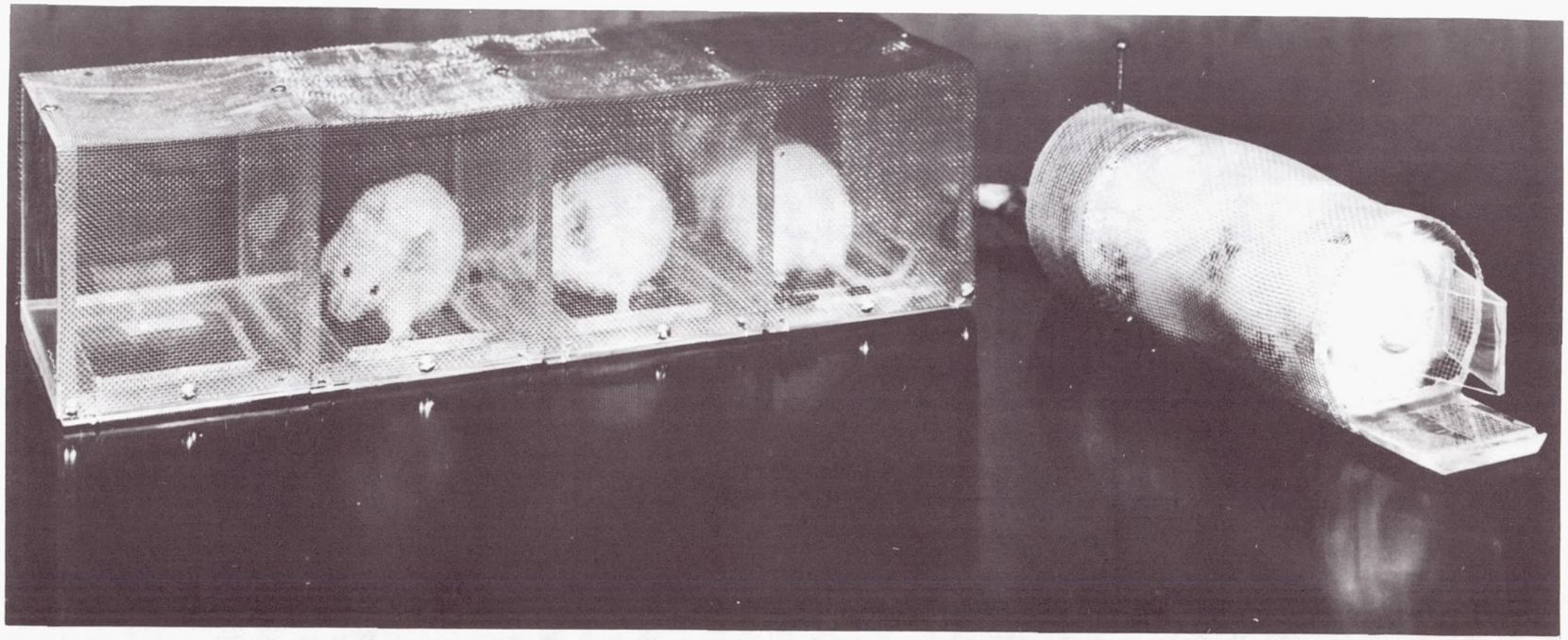


Figure 12. Restraining Devices for Mice and Rats for Oxygen Consumption Measurements in the CES-5.

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 (HeP, N_2P, HeF_2, N_2F_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 (Guildford, 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 fluxtuations were repeated, regardless of the values. As before, repeat experiments were done in pairs and in random order.

D. Results

1. Rats

Oxygen consumption data from rat experiments is summarized in Table IX and presented graphically in Figure 13. A statistical comparison of the various situations is given in Table X.

TABLE IX

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 In Air	He Rats O_2 ml/kg/min, STPD In HeO_2	N_2 Rats O_2 ml/kg/min, STPD In Air	N_2 Rats O_2 ml/kg/min, STPD 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	

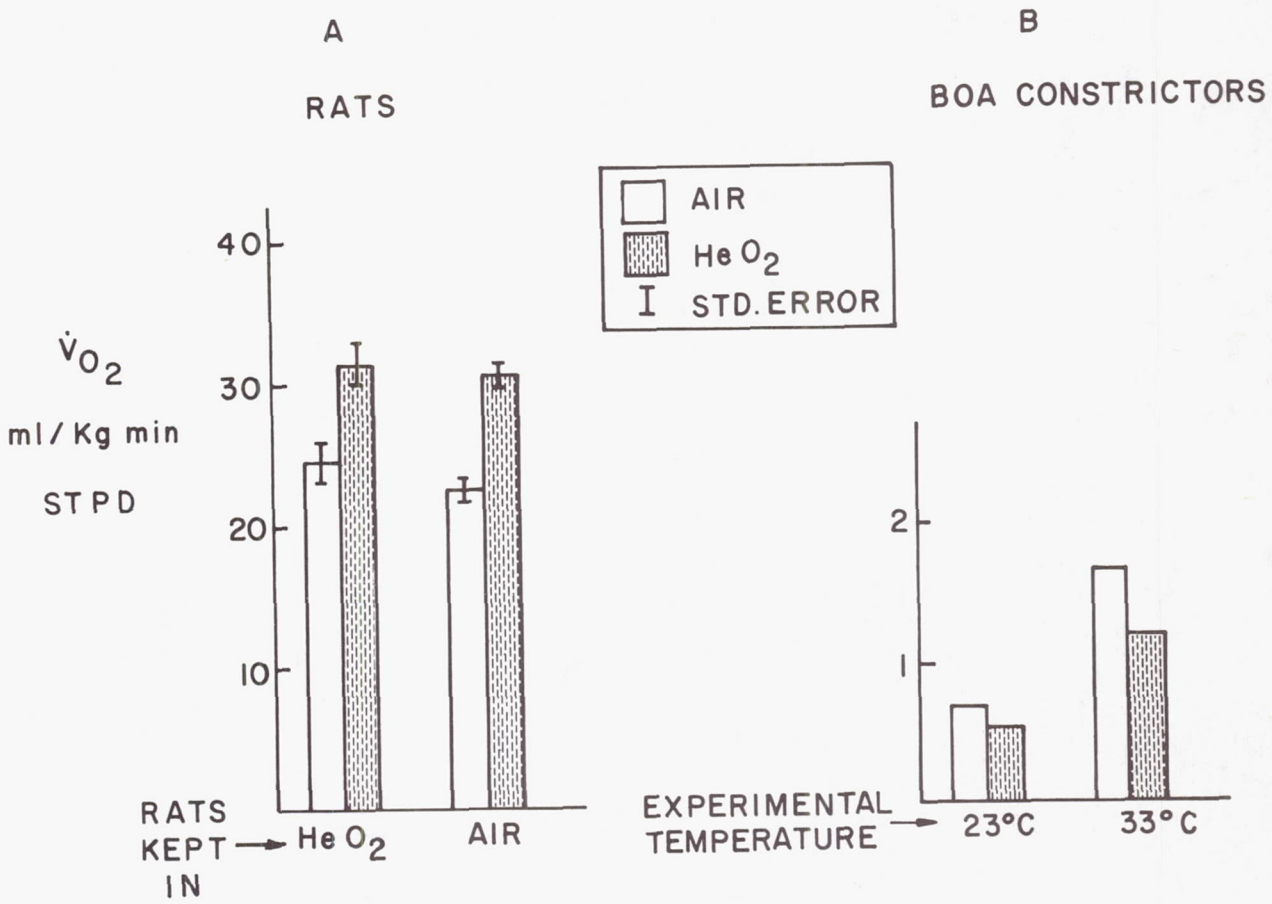


Figure 13. Oxygen consumption of Rats and Boa Constrictors.
A: Comparison of response of air-raised (right) and helium-raised (left) rats with respect to their response to the HeO_2 atmosphere.
B: Responses of boa constrictors to air and HeO_2 at two different temperatures. Boas were kept in air.

Table X

STATISTICAL SUMMARY OF OXYGEN CONSUMPTION DATA, RATS

Comparison	t	df	P	% Difference in $\dot{V}_{O_2}$
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	-

Figure 13A 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 XI and Figure 14. The individual entries represent three successive 10-minute measurement periods. A number of these categories were compared statistically using the paired t-test; statistical results and per cent differences are given in Table XII.

TABLE XI

OXYGEN CONSUMPTION OF MICE IN 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	54.8	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	79.0	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 ± s.e.		50.2 ± 2.0	66.3 ± 3.2	48.5 ± 2.3	59.1 ± 2.7	53.6 ± 2.8	77.1 ± 5.1	62.5 ± 3.4	86.5 ± 3.6
$\bar{x}$ Weight in grams		48.5		48.5		34.7		32.3	

Table XII

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
He O_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
He O_2	0.243	35	>0.05	-
He P vs He F_2				
Air	0.948	17	>0.05	He F_2 7% > He P
He O_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
He O_2	6.107	17	<0.001	N_2 F_2 46% > N_2 P
Air vs He O_2				
He P	4.282	17	<0.001	He O_2 32% > Air
N_2 P	2.372	17	< 0.05	He O_2 22% > Air
He F_2	3.959	17	< 0.001	He O_2 44% > Air
N_2 F_2	4.874	17	< 0.001	He O_2 38% > Air
All He mice	5.585	35	< 0.001	He O_2 38% > Air
All N_2 mice	4.373	35	< 0.001	He O_2 31% > Air
Order of exposure				
He P in Air	0.941	8	>0.10	-
He P in He O_2	0.805	8	>0.10	-
N_2 P in Air	0.466	8	>0.10	-
N_2 P in He O_2	0.030	8	>0.10	-
He F_2 in Air	1.000	8	>0.10	-
He F_2 in He O_2	0.112	8	>0.10	-
N_2 F_2 in Air	0.315	8	>0.10	-
N_2 F_2 in He O_2	0.312	8	>0.10	-
All He mice in Air	0.039	17	>0.10	-
All He mice in He O_2	0.046	17	>0.(0	-
All N_2 mice in Air	0.198	17	>0.10	-
All N_2 mice in He O_2	0.016	17	>0.10	-

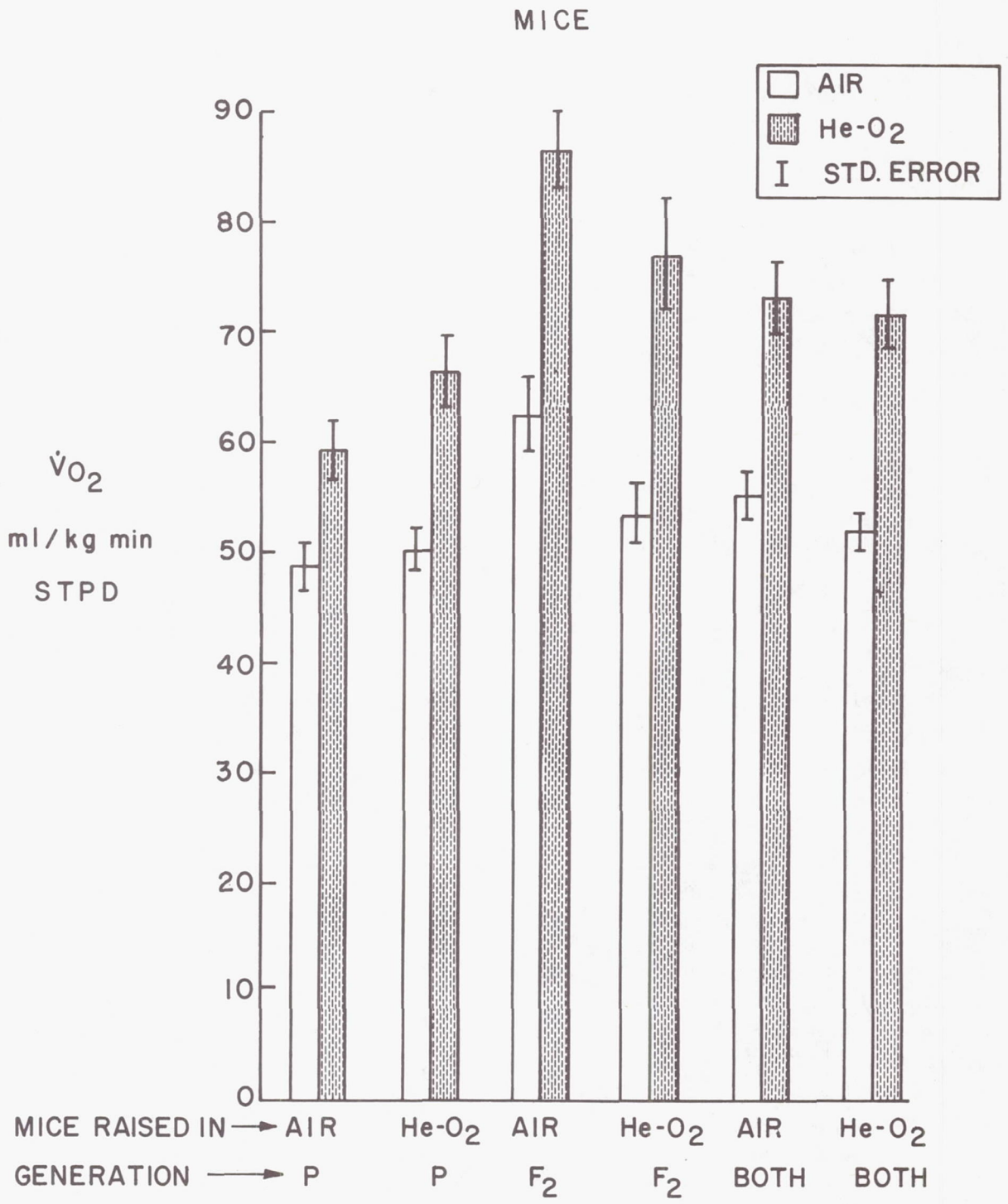


Figure 14. Oxygen consumption of mice. The open bars show the mean oxygen consumption of mice in air, and the shaded bars the consumption of the same mice in HeO_2. Order of presentation of the gases was mixed, and had no effect.

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 14, 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.

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 system. The so-called "cold-blooded" animals orheterotherms 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 beobtained 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° and 33°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 XIII and on Figure 13B.

TABLE XIII

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
x	0.70	x	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
x	1.68	-	1.21
weight, kg	1.56 and 1.21		
internal temperature	33.0°C and 32.8°C		

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, Bruemmer, and Doebbler, 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 11) 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%.

V. CES-4: A SEMICLOSED ENVIRONMENTAL SYSTEM FOR MAINTAINING SMALL ANIMALS IN VARIOUS GASEOUS ATMOSPHERES

A. Summary

A semiclosed system was designed and constructed to permit the long-term exposure of small laboratory animals to different inert gas/oxygen mixtures at atmospheric pressure. The system automatically maintains two animal chambers, each of approximately 300 liters volume, in parallel at identical oxygen partial pressures, total pressure, temperature, and humidity. An alarm system signals in case a malfunction occurs in any of the subsystems considered vital. Normal personnel attention required for the maintenance of the system is about 1-hour per day.

The system compensates automatically for any change in oxygen demand. Oxygen and inert gas are supplied by flow meters at a rate of about three chamber volumes per day. Additional inert gas or oxygen is independently supplied to each chamber as needed, by means of a polarographic detector and electronically controlled solenoid system. Carbon dioxide is continuously removed by a Baralyme scrubber in a gas recirculation loop. Temperature and humidity are controlled by a water-cooled heat exchanger and an electric heater. Because of the continuous flow of inert gas, the accumulation of ambient nitrogen is kept at a minimum--less than 0.2% in our experience.

Animals maintained within these chambers are easily handled through gloves. Locks allow transfer of food, animals, or cages into and out of the chambers with a minimum of contamination of the special atmospheres.

The system operated without unscheduled interruption during this six-month experiment in which three generations of mice were raised in a helium-oxygen and a parallel air environment.

B. Description

1. Design Concepts

This section deals with the design, construction, and performance of the chamber system used for the long-term study of the effects of helium as a substitute for nitrogen in the gas environment.

The task was to expose two groups of small animals to environments identical in every way except for the nature of the inert gas. One chamber was intended to expose control animals to a normal air environment, but to equalize any deficiencies in the control system of the experimental chamber we chose to use an artificial nitrogen-oxygen mixture in this chamber.

The system consists of two plexiglass chambers of about 300 liters volume each to which oxygen and inert gas are constantly supplied and from which CO_2 and excess water vapor are constantly removed. The chamber designated "A" was supplied with the experimental atmosphere, 80% helium and 20% oxygen. Chamber "B" was supplied with nitrogen and oxygen and contained animals used as controls. The normal environmental parameters of total pressure, oxygen and CO_2 partial pressures, humidity and temperature were closely maintained, with the inert gas (helium or nitrogen) representing the only difference between the environments in the two chambers.

Since the experiment was designed as a 6-month around-the-clock exposure, emphasis was placed on automatic operation of the system, for minimum personnel attention. An alarm system was designed and installed for protection in case of failure of those subsystems considered to be vital.

Basically, the apparatus is a semiclosed environmental system in which oxygen and helium are supplied at a rate of 3 to 4 chamber volumes per day to Chamber "A" and an equivalent amount of nitrogen and oxygen to Chamber "B." A gas-composition-regulation-system modulates the amount of gas flow to each chamber and controls the composition of the atmosphere. In a gas recirculation loop, CO_2 and excess humidity are removed and heat is added or removed as necessary. In the present experiment, oxygen was maintained at 150 ± 10 mm Hg in both chambers. An overall view of the entire system is shown in Figure 15, and a flow diagram is shown in Figure 16.

2. The Chambers

The original chambers which formed the basis of the system were purchased from Manostat Corporation as nonvacuum glove boxes. Their overall dimensions are 36 in. long x 21 in. wide x 22 in. high, and they are constructed of 1/4-in. Plexiglass. They feature an air lock 12 in. in diameter x 12 in. long which is capable of supporting a vacuum and therefore provides a means of transfer without air contamination. The

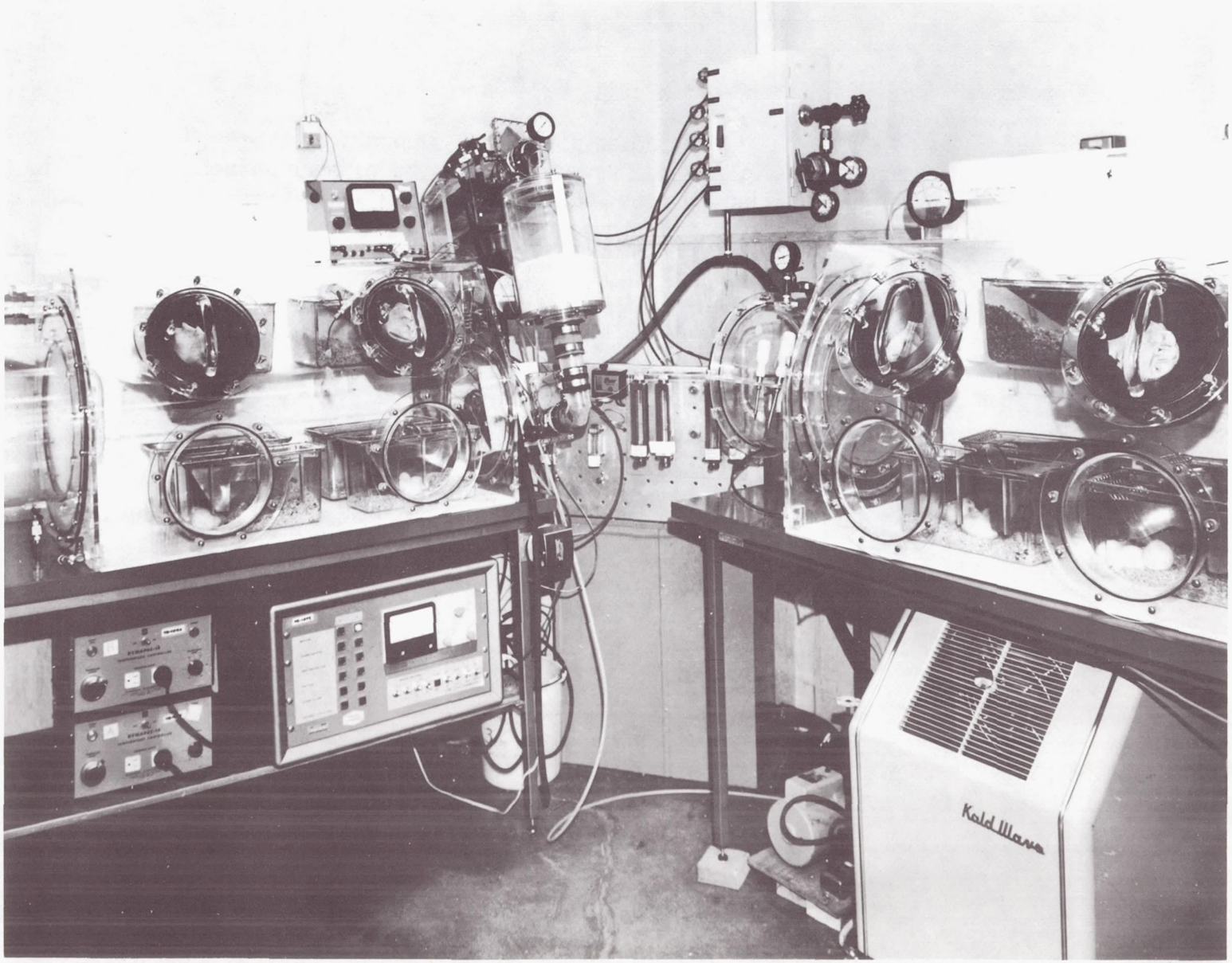


Figure 15. A General View of Controlled Environmental System 4.

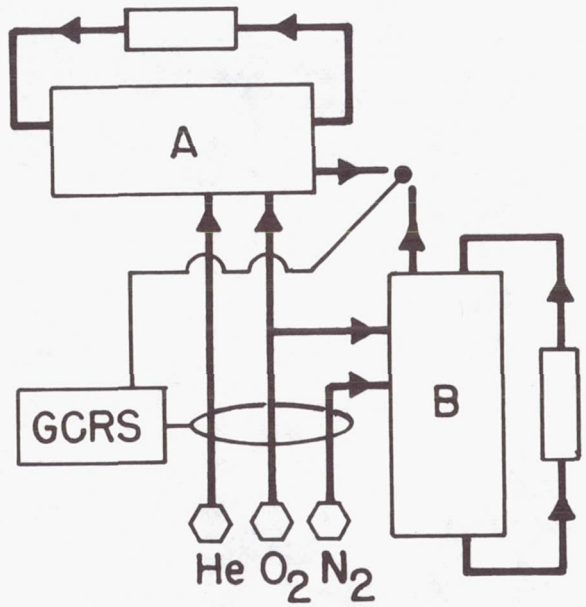


Figure 16. Overall Flow Diagram for the CES-4 System. The two chambers with their recirculating loops are shown, and the gas consumption regulating system as it relates to supply gas. The black dot represents the oxygen detector.

glove boxes came supplied with a recirculating gas loop external to the basic plexiglass box, consisting of an 80 cfm centrifugal blower, a 6-in. diameter by 8-in. long cylindrical filter unit piped with 2-1/4-in. I.D. plastic tubing and rubber hoses. The flow of gas returning to the chamber is uniformly distributed by means of a graded manifold. The handling of equipment or animals inside the chamber was made possible by means of two neoprene gloves mounted on heavy rubber bellows and located on the lower front of the box. All the covers (i.e., for gloves, air lock, and filter unit) were provided with "O" rings and were fastened to metal inserts within the plastic by means of machine screws. The original chambers were subjected to extensive modifications and additions. The gloves were repositioned at a higher level to facilitate the handling of animal cages. Major modification was done to the gas recirculation loop, which will be treated in detail in Section V, B4. below. Vacuum and pressure gages as well as electrical, water, and gas penetrations were added to the system. (See Figure 17)

3. Gas Composition Control System

a. Makeup and Control

Three to four changes of chamber gas volume per day were chosen in order to provide a relatively stable oxygen level and to eliminate trace contaminants. This volume of gas was constantly delivered to the chamber and an identical volume was simultaneously exhausted. This amount of flow was not sufficient to remove carbon dioxide or humidity, so a recirculating system was needed to control these parameters. To keep a constant oxygen concentration with this system would have required a rather high flow of properly mixed gases. The use of premixed helium-oxygen would have been prohibitively expensive, and we were not aware of a sufficiently accurate continuous mixer, so we chose to set up a control system with feedback.

The supply of oxygen and inert gas to each chamber was set with manual needle valves and rotameter flowmeters. Each of these valves was paralleled by a solenoid-controlled needle valve which acts as a "trim" in adjusting the flow of gas. (See Figure 18) The solenoids were controlled by a gas composition regulation system which selectively opened the bypass flow as needed. Flowmeters and floats were selected appropriately for the gases being used. Flows were set initially at 500 ml/min for inert gas and 125 ml/min for oxygen, and adjusted as required by the performance of the control system. The flowmeter panel is shown in Figure 19. The gas supply, at 20 psi pressure, was piped into the building from outdoor manifolds.

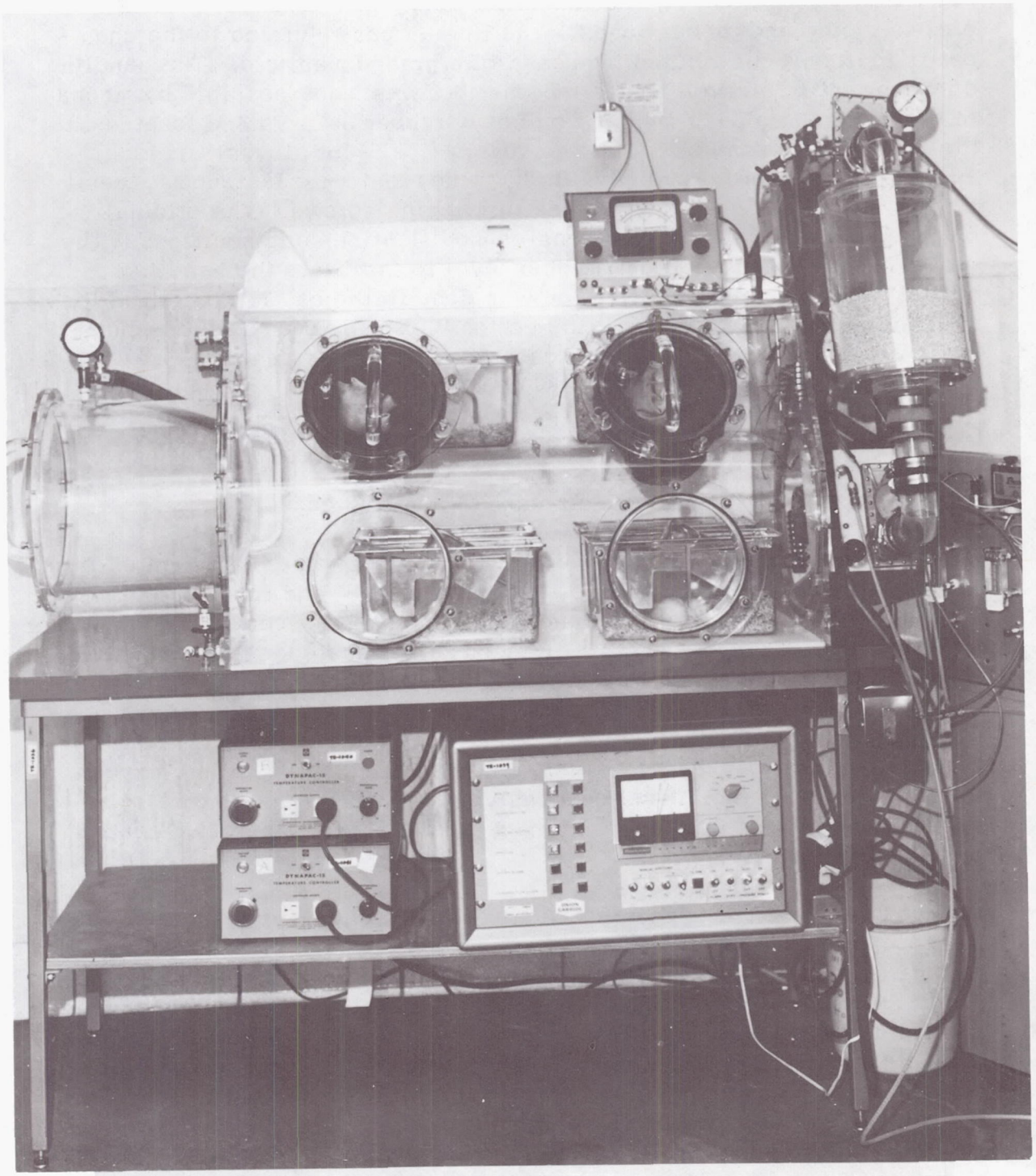


Figure 17. CES-4. Chamber after all modifications were completed.

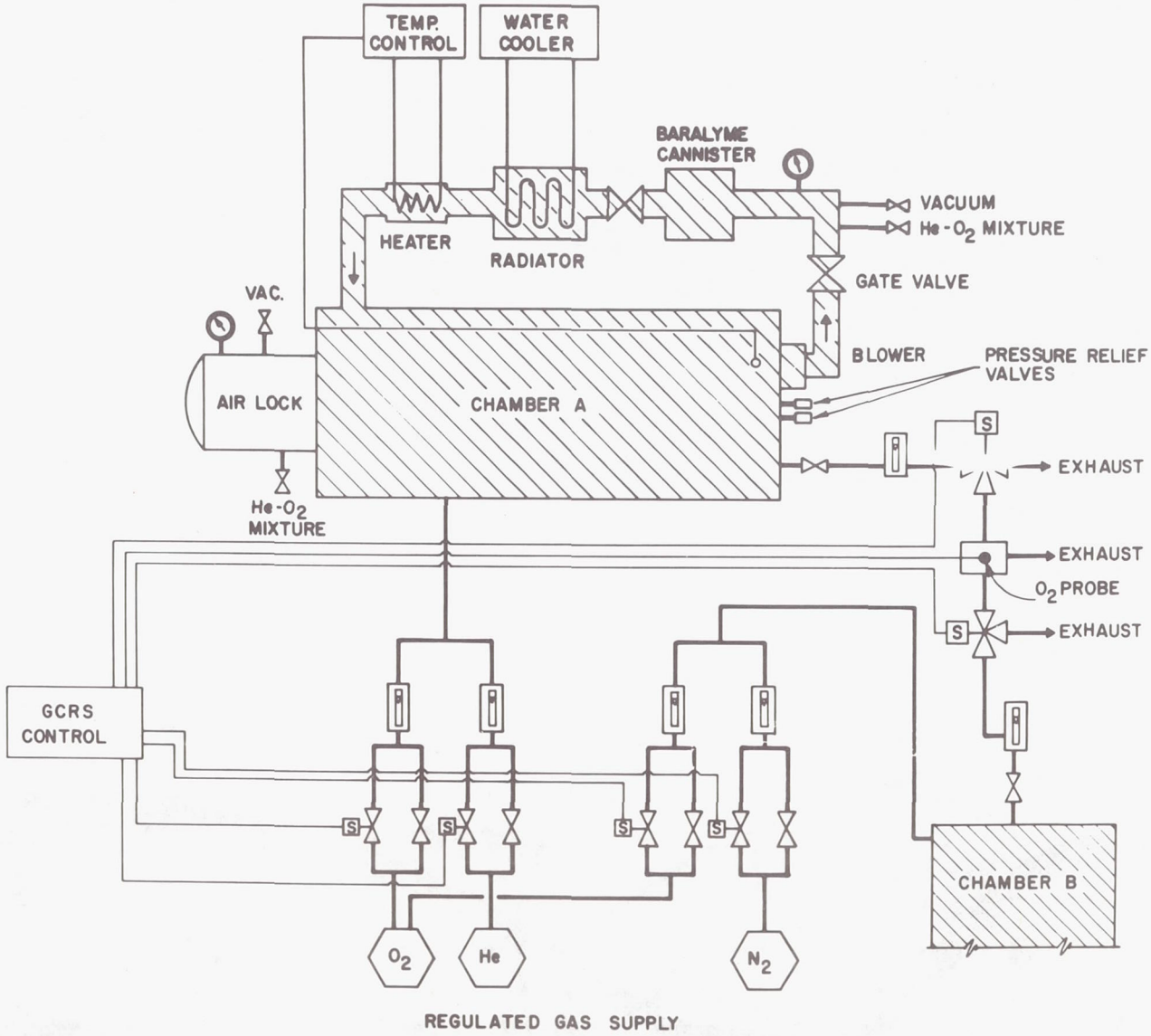


Figure 18. Detailed Schematic of the CES-4. Recirculation loop and accessories are identical for both chambers.

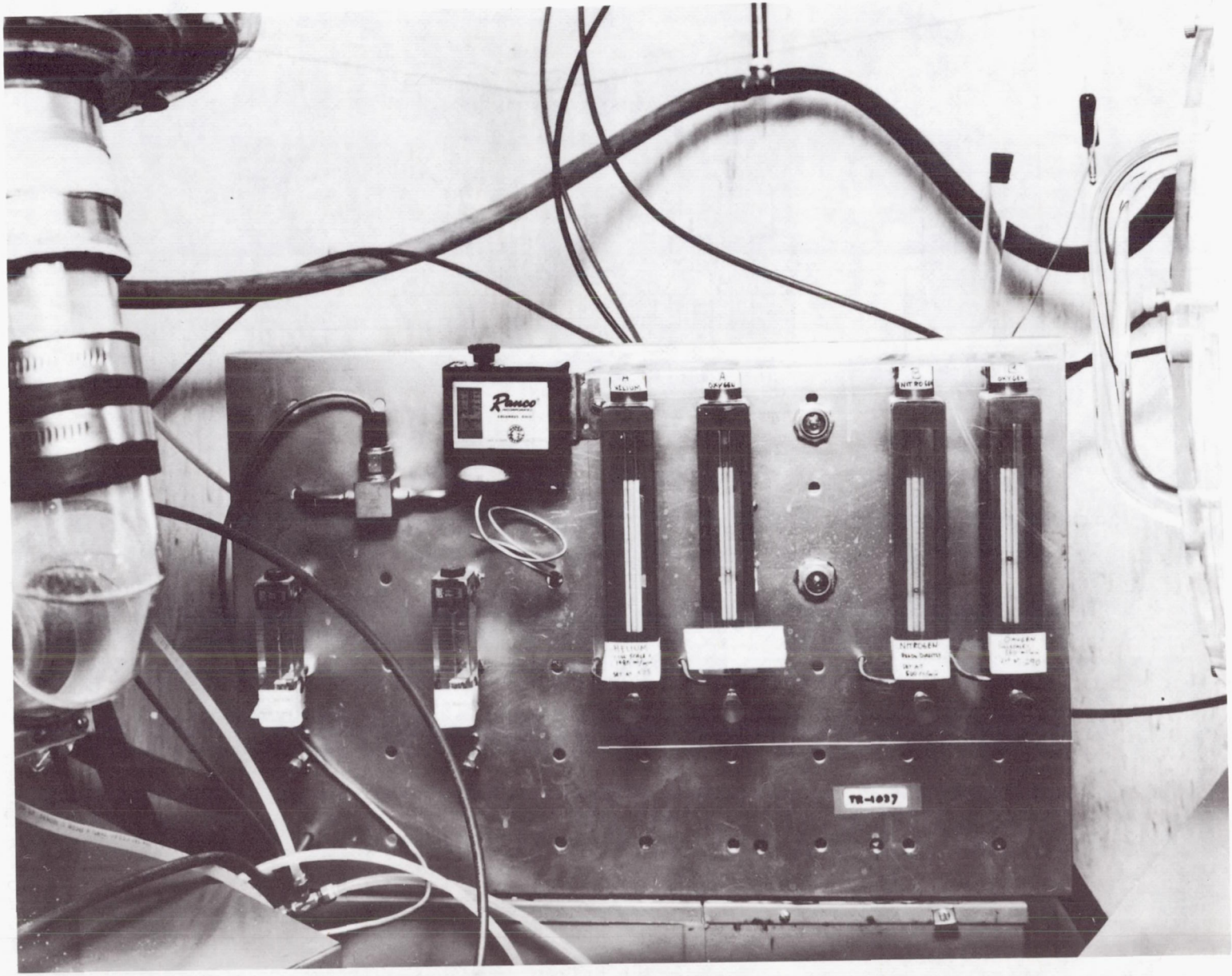


Figure 19. CES-4. Front view of Flow Meter panel.

The heart of the gas composition regulation system was a Beckman Model 777 polarographic oxygen analyzer. This unit was modified by replacing the meter with a 503-L double set meter relay (Assembly Products, Inc.) which could be set to signal deviations from the selected band.

The analyzer was installed so that the exhaust of both chambers alternated through the oxygen sensor cuvette. This was achieved with the aid of a recycling timer and two 3-way solenoid valves. The oxygen electrode was fitted into a specially built brass cuvette with two gas inputs and an exhaust open to the room. The inputs were connected to the two chambers via 3-way solenoid valves. When one of the valves was energized, sampling gas from that chamber reached the oxygen probe; the other solenoid was simultaneously de-energized, and exhausted the sample from that chamber to the room.

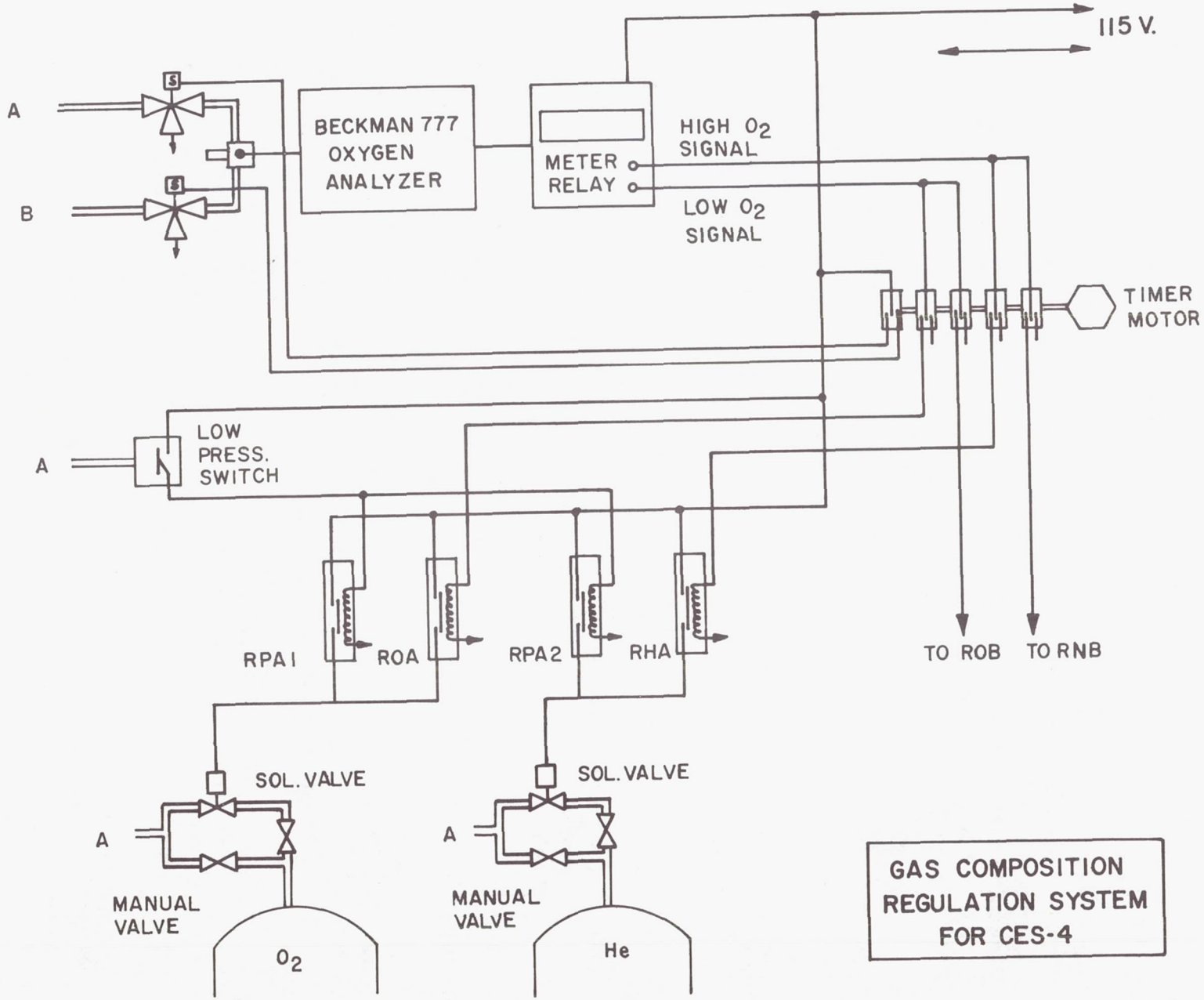
The timer (Industrial Timer Co.) has five circuits with programmable cams. The cams were programmed such that circuit one was used to alternate the exhaust of the chambers through the probe and the other circuits connected the output of the meter relay to the proper relay coil in synchrony with the oxygen measurement (Figure 20). A 1-minute disconnect was allowed at each chamber switching to avoid transient misreadings. The total cycle period was 15 minutes.

b. Pressure

The total pressure inside the chambers was maintained from 7 to 10 mm Hg above atmospheric to reduce contamination of the helium by atmospheric nitrogen.

A maximum pressure of 10 mmm Hg above atmospheric was maintained by a Foregger pressure relief valve (Cat. No. 7-933-120). This valve released the internal gas to the outside atmosphere when the differential pressure rose above a certain adjustable level. Two such valves were installed on each chamber. One was set at 10 mm Hg differential pressure and the other was set at 20 mm differential pressure in case that the first one failed to actuate.

The minimum pressure point was controlled by a Dwyer differential pressure switch. If, due to excessive leakage, the pressure in the chamber decreased to 5 mm Hg above atmospheric, a contact closed, energizing two relays. The first relay energized the solenoid bypass valve on the oxygen line and the second opened the helium bypass. The result was an increase of pressure inside the chamber. When the pressure reached the minimum level, the pressure switch opened, stopping the corrective action.



GAS COMPOSITION
REGULATION SYSTEM
FOR CES-4

Figure 20

c. Console

A control console (Figure 21) houses the electronic components of the gas composition regulation system. These include the oxygen analyzer, meter relay, the recycling timer, the relay network, and an Acromag relay used for a low oxygen alarm.

Monitor lights on the panel display the conditions of the chambers in reference to gas composition, low differential pressure, and oxygen and flow alarm. They indicate which chamber is being monitored by the oxygen analyzer, and when oxygen or inert gas is being added to either chamber. Two red lights for each chamber indicate that oxygen partial pressure is critically low and that there is no gas recirculation flow.

Switches for extra gas addition permit the manual addition of either oxygen or inert gas at any moment to either chamber. Switches were also provided to inactivate the gas composition and alarm systems during maintenance and routine animal handling.

4. Recirculation System

a. Carbon Dioxide Removal

Because of its ready availability and ease of handling we chose Baralyme, a granular CO_2 absorbent, to remove metabolic CO_2. The canisters which were supplied with the chambers were replaced with ones of a larger capacity in order that a larger animal population could be supported. As a design criterion, we chose the widely accepted P_{CO_2} level of 3.5 mm Hg as a practical upper limit, assuming that CO_2 levels lower than this would have no effects on the outcome of the study. For a Baralyme efficiency of 95%, a flow of about 10 liters per minute was calculated to handle the CO_2 production of our animal population without exceeding this value. The blowers supplied with the chambers were rated as more than adequate for this flow. However, the maintenance of temperature and humidity required a larger flow and this proved to be the limiting factor in the system.

The Baralyme had to be changed daily when the system was operating at capacity population, but with only a few animals it could be changed less frequently. To facilitate changing the absorbent without allowing room air to infiltrate, gate valves were installed so that the canister could be isolated. These 2-1/2-in. valves were constructed in our shop; each consisted principally of a sliding plate and O-ring seals. After refilling the canister of Chamber A, the canister and tubing between the valves was evacuated three times and refilled with a premixed helium-oxygen mixture. We felt that this was not necessary in the case of the chamber filled with air.

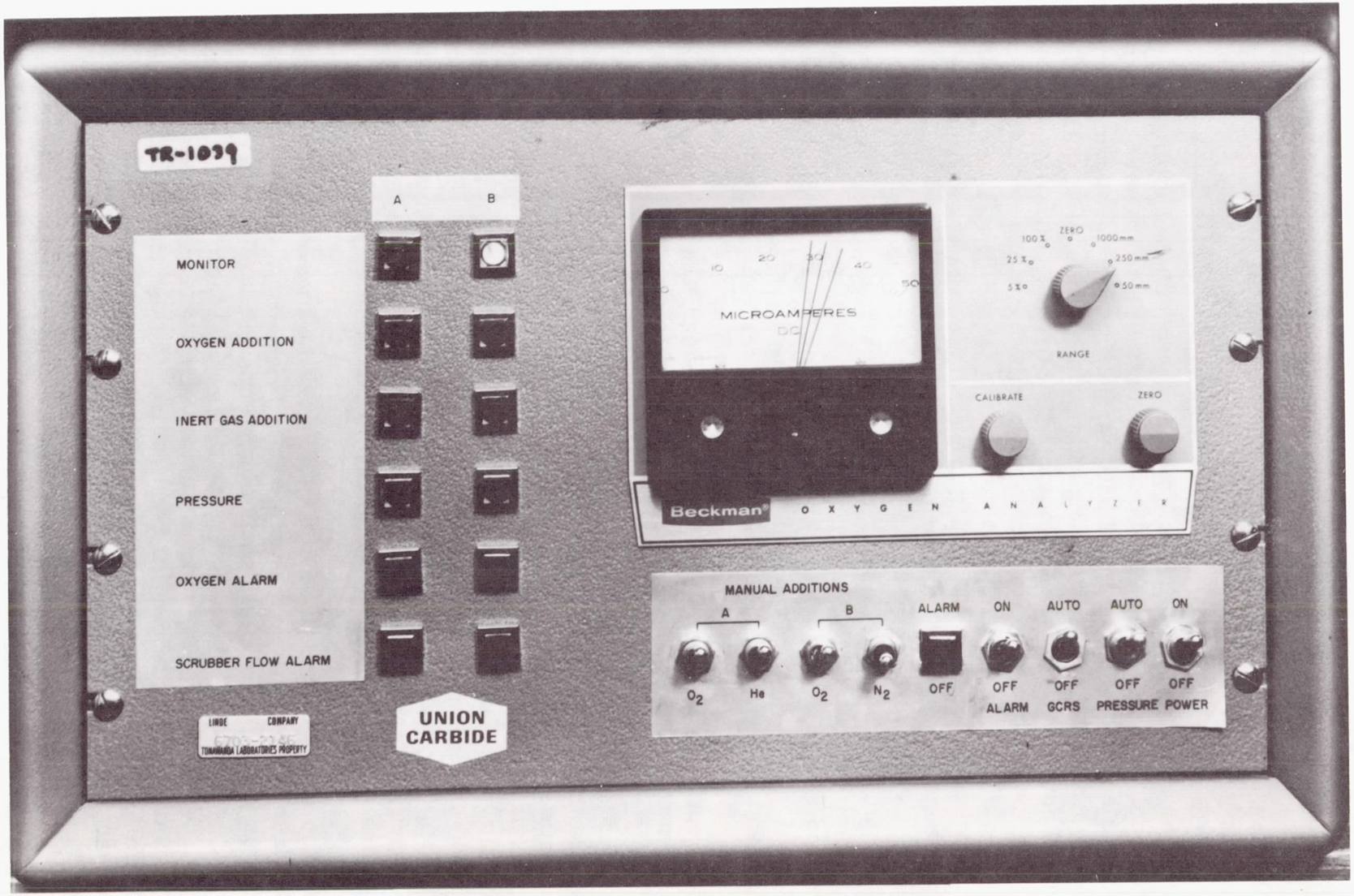


Figure 21. Front view of CES-4 Control Console.

A view of the canister and recirculating system is shown in Figure 22.

b. Temperature and Humidity

The experimental design called for both chamber atmospheres to be identical in all respects except one, but in addition posed the requirement that conditions be as normal as possible. Thus a temperature of 24°C was sought, and a relative humidity between 40 and 60%. The design philosophy we selected to accomplish these conditions was a familiar one used in commercial and industrial applications. Humidity was controlled by cooling the chamber gas to the desired dew point, and temperature was controlled by rewarming the gas stream. Humidity control with silica gel was considered, but thermal balance calculations revealed that considerable cooling was required, and this made our chosen control philosophy practical.

Cooling was accomplished by a Temprite water chiller similar to that of an ordinary drinking fountain. This was modified with a thermostat which provided a greater range of temperatures. The capacity of our unit was 2500 B.t.u./hr. and this proved to be adequate for humidity control in both chambers. Coolant (water) was circulated by a parallel piping system to a heat exchanger (a small automobile heater radiator) located in each chamber gas stream, downstream of the Baralyme. Condensate drained into a 1-liter flask which was emptied daily.

Reheating of each gas stream was accomplished just downstream of the radiator with a heating unit from a hair dryer connected to a Dynapac-15 solid state proportional controller. A separate heat controller was used for each chamber, but a single water temperature served for both. Individual humidity adjustment could be accomplished by throttling either coolant or gas flow to the individual heat exchanger. Since the same dew point was wanted in both chambers, this was not necessary. For our purposes, the reading of a Wayne-Kerr gold element resistance hygrometer was accepted as desired relative humidity, and this was backed up by dry bulb dew point readings taken in the gas stream. Neither of these methods takes into consideration the different physical properties of the gases, but there were no notable disagreements and since a fairly broad range of humidity was acceptable, no further efforts to measure humidity were made.

The physical properties of the two different inert gases made a major difference in another way, however, The centrifugal blowers which delivered 6.4 cfm of air would move only 4.2 cfm of the helium-oxygen mixture. To maintain comparable flows, we had to install a larger blower on Chamber A.

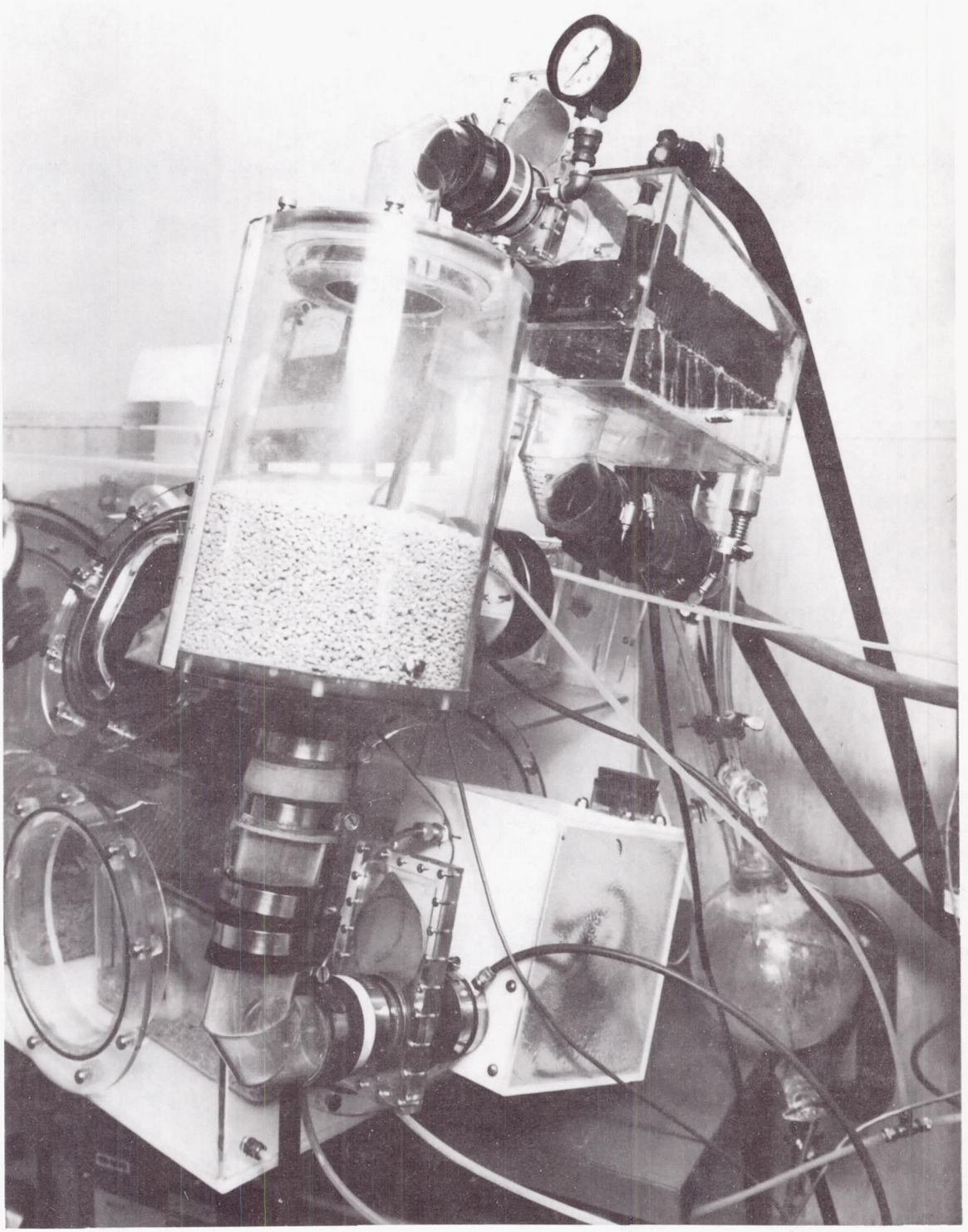


Figure 22. Gas recirculation system of Chamber A, CES-4, showing blower box, CO_2 scrubber, radiator, heater, and water collection flask. The blower is housed in the square box near the bottom of the system.

As described here, the system proved somewhat inadequate in removing the heat from the helium chamber. The larger blower on the helium chamber was effective for all but a maximum animal load. During periods when the population was large, Chamber A tended to heat up to 26°C. Additional flow would have prevented this, but at these times more Baralyme was required, and this tended to restrict the flow. Turning down the thermostat on the water chiller would have lowered the dew point as well as the temperature. The solution was to add a small air conditioner to the room and remove excess heat by maintaining a lower room temperature. This returned control to the Dynapac units.

5. Alarm System

In addition to an emergency power unit set to take over in the event of a power failure, we felt that other malfunctions might threaten the animals and hence the experiment. We planned an alarm system which we hoped would alert someone soon enough to correct the condition in time to prevent disaster.

Conditions which we felt represented the greatest threat were low oxygen and inadequate gas circulation, and an alarm was devised to detect each of these situations.

The low-oxygen alarm is an Acromag magnetic amplifier relay connected to the recorder output of the oxygen analyzer. It was set to trigger at 75 mm Hg. The flow alarm consists of a pressure switch (Dwyer No. 1822-5) and a relay for each chamber. The pressure terminals of the switch were placed between the high-pressure side of the blower and the chamber. Any time the blower stops, the pressure across the switch vanishes, closing the switch and energizing a relay. Three such relays (one from each pressure switch and one from the oxygen alarm) were connected to an alarm circuit with one of their normally closed contacts in series. This circuit was wired so that if any of these three contacts opens, it will signal ADT, a commercial alarm signal company. The other pole of the relay was used to produce an audible signal whenever the alarm is triggered. There are switches to disconnect the alarm during chamber servicing and maintenance. Red lights on the front panel of the control console pinpointed the source of the alarm. No emergencies which would have set off the alarm occurred during the experiment.

C. Performance

This section presents an overview of pertinent data observed during the operation of CES-4.

1. Routine Maintenance

Certain procedures were systematically or routinely done in order to effect a smooth operation and to prevent operational problems. The following schedule indicates daily, weekly, and aperiodic procedures.

Daily Procedures

a. Chamber temperatures were measured and recorded twice daily.

b. Gas samples were aspirated with a syringe and then analyzed for CO_2 content.

c. The color of the water in the collector flasks was noted to indicate the presence of ammonia. Ammonia, dispersed in the circulating gas, chemically reacted with the copper surfaces of the radiator, giving a blue color to the water in the collector flask. Initially, the amount of ammonia was calibrated by bubbling the water through a saturated boric acid solution and then titrating with 0.01 M HCl. This value was then correlated with the intensity of the color present and arbitrarily assigned values from 0 to +4.

Arbitrarily Assigned Score	p.p.m. Ammonia
0	30
+1	75
+2	115
+3	150
+4	200

d. Helium, oxygen, and nitrogen supplies were checked for pressure and content.

e. Oxygen analyzer membrane was checked for integrity and calibrated to oxygen content of room air.

f. Ambient barometric pressure was recorded twice daily.

g. Water bottles were refilled and water consumption measured every other day.

h. Waste water in the collection flasks was discarded daily.

i. Baralyme in canisters of Cambers A and B was changed daily.

Weekly Procedures

a. Feed was measured and supplied once weekly.

b. All cages were cleaned and bedding changed twice weekly. A treated sawdust bedding was used, and spread one inch deep on the bottom of each cage.

c. Gas samples were extracted twice weekly for gas chromatographic nitrogen determination.

Various component temperatures were taken aperiodically and are listed below in the data section.

2. Data Accumulated From Routine Maintenance Procedures

Parameter Measured	Average Value	Daily Fluctuation	Range
Chamber temperature	24°C	± 1°C*	21.0°C-29.5°C
CO_2	0.5%	0.05%	0.1-0.6%
Ammonia	+1	0.5	0- +4
Barometric pressure	743.7 mm Hg	0.1 mm Hg	741-752.5 mm Hg
Chamber N_2	0.5%	± 0.5%	0.05-1.0%

* Standard deviation: Chamber A: ± 0.9°C (287 measurements);
Chamber B: ± 1.0°C (269 measurements).

Aperiodic Temperature Measurements

Item	Average Temperature °C
Water leaving cooler	8.7
Water leaving radiator (entering cooler)	12
Gas leaving radiator	10.5
Gas entering chamber	14
Gas inside chamber	24
Room temperature	23.3

3. Logistics

Below is a list of items which were required for completion of this study and the quantity used per unit time:

Item	Quantity/Unit Time
Mice	527*
Rats	28*
Feed	16 lb/wk
Bedding	20 lb/mo
Baralyme	10 lb/day/chamber
Helium	1140 liters/day
Oxygen	910 leters/day
Nitrogen	1170 liters/day

* Total number of animals used in this study

D. Discussion and Critique

This system performed as specified and enabled us to complete this 6-month experiment as planned. A number of problems came to light and had to be solved in turn. Some defects of design are evident and can be corrected for future experiments.

The major problem was cooling. As mentioned before, at the time when the metabolic load was the greatest and the maximum animal heat had to be removed, the Baralyme requirements were also greatest and under these conditions the blowers proved to be inadequate. The blowers were located inside the chambers, an arrangement which minimized leaks but which resulted in both the heat and the kinetic energy of the motors being contained within the system. Solutions to this problem would have been to locate the blowers outside of the chambers or to provide cooling coils around the motors. One additional problem caused by the blowers being inside was that they cannot easily be lubricated, and if lubrication was inadequate the heat problem became even greater.

A second source of trouble was leaks. We did not eliminate these well enough at the beginning, and never modified the system so that it did not have an appreciable leak rate. Better leak elimination would have made control more precise and saved gas.

Another main problem was the cost of CO_2 absorbent for long-term experiments. One approach to this would have been to provide dual canisters and sequence them, allowing the partially spent absorbent to contact the incoming gas. This was not likely to be effective at the CO_2 levels we have maintained. Another way would have been to use a liquid scrubber, but this requires considerably more energy for gas circulation.

The alarm system was not ideal. The low oxygen alarm depended on proper functioning of the oxygen controller. A redundant system would have been preferable. Perhaps a simple pressure switch on the oxygen supply line would have also helped. The pressure-drop method of signaling flow failure could not detect a block, such as a valve left closed. A better method would have been a flag switch in the gas stream. In view of the difficulties we have had with temperature, perhaps a temperature alarm would have been desirable.

During the first month of the experiment, improper procedures for the changing of bedding in Chamber A caused an appreciable rise in chamber nitrogen content immediately after each bedding change. At one such occasion, a P_{N_2} of 0.2 atm. was detected. Procedures were amended, however, so that for the balance of the experiment bedding changes produced a transient rise of P_{N_2} to no more than 0.01 atm.

Specific problems which arose should be mentioned. The major one was an overpressurization during maintenance, resulting in the cracking of the wall in Chamber A with a resultant loss of the helium environment for about 4 hours. This was apparently the result of an improperly set flow valve. Another time, flow was restricted by the accumulation of water at a low point in the recirculation tubing. Once the sampling flow was shut off and one chamber experienced a drop in P_{O_2} to below 100 mm Hg. In all these cases there were deviations from the experimental conditions, but these were temporary and experimental analyses and measurements were suspended for several days following the incident.

One secondary feature of the system occurred by accident. During the early days before we had established proper procedures for maintaining control over ammonia formation (by the frequent changing of bedding), we noted that the condensate water had a blue color that seemed to correlate with ammonia as determined by chemical analysis. This we found to be due to the formation of copper-ammonia complexes with the brass in the heat exchanger. As a result, we were able to judge the ammonia levels by the color of the condensate and did not need chemical monitoring.

Despite its faults, this is a reliable and versatile system. It is worth mentioning the tremendous saving in statistical effort and replication that was made possible by having a control chamber maintained in parallel with the experimental one. About the only defect here was the possibility that an infection might invade one chamber and not the other.

This type of system can be used for other environmental problems, such as chronic hypoxia or long-term exposure to CO_2. It could be used for slightly larger animals or even perhaps for microorganisms or plants.

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APPENDIX

Table I

Pathologic Evaluation of Mouse Tissues

Mouse No.	Age Days	Weight g.	Gas and Generation	Findings
9	29	39	He - P	Kidney - a few scattered round cell foci. Adrenal - large number of vacuoles and vacuolated cells at the cortical medullary junction, this is a unique finding but its significance is unknown. In addition, there are many large foam-type cells in this same area which have been seen by this pathologist on other aged mice. Pink casts are present in the renal tubules in small amounts.
10	29	43	He - P	Kidney - a few scattered round cell foci. Adrenal - similar to the first with some vacuoles at the cortical medullary junction and some large foam-type cells in the same area.
11	34	34	N_2 - P	Adrenal - only a few vacuoles at the C. M. junction and only a few foam-type cells in this area.
12	34	39	N_2 - P	Adrenal - large number of foamy-type cells and relatively few vacuolated cells. One gets the opinion that these foamy-type cells in the Adrenal Gland are multi-nuclear phagocytes undergoing some type of hydropic change.
28	68	38	He - F_1	Adrenal - N. R., in that, no vacuoles and no foamy phagocytes are present at the cortical medullary junction.
29	68	34	He - F_1	Adrenal - N. R.
30	68	38	N_2 - F_1	Adrenal - N. R.
31	68	38	N_2 - F_1	Kidney - one or two scattered casts on each Kidney. Adrenal - N. R.
98	59	36	N_2 - F_2	Kidney - a few scattered round cell foci. Some glomeruli have a cuboidal lining. Liver - some variation in staining intensity of scattered cord cells, this may be an artifact. Adrenal - N. R.

Table I Continued

Mouse No.	Age Days	Weight g.	Gas and Generation	Findings
99	59	36	$N_2 - F_2$	Liver - some variation in nuclear size. Adrenal - N.R.
100	59	37	$He - F_2$	Adrenal - N.R. Kidney - a few glomeruli with cuboidal linings.
101	59	35	$He - F_2$	Adrenal - although only a small section of adrenal present it does include both the medulla and cortex and no lesion is observed.

Table II

Metabolite Measurements on Mouse Liver Tissue

Data of J. Kissane, Illinois Inst. Tech. Res. Inst. (1967)

	μ mole/gm tissue	
Compound	Nitrogen Environment	Helium Environment
ATP	2.84±0.51	2.13±0.45
ADP	1.01±0.26	1.12±0.32
AMP	1.29±0.32	1.33±0.26
NAD	0.543±0.143	0.438±0.150
NADH	0.048±0.008	0.076±0.018
NADP	0.124±0.023	0.109±0.042
NADPH	0.693±0.175	0.835±0.234
Pyruvate	0.172±0.132	0.207±0.068
Lactate	7.42±1.51	5.85±1.44

(No statistically significant differences between nitrogen and helium group mice were found at $P < 0.05$.)