MONOGRAPH OF GASES IN FOODS #63

Monograph on **Gases In Foods** #63 10/16/74

M O N O G R A P H

O N

G A S E S I N F O O D S

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INFORMATICS INC.
6000 Executive Boulevard
Rockville, Maryland 20852

GASES IN FOODS

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GASES IN FOODS

Summary

Five gases, butane, helium, nitrogen, nitrous oxide and propane are included in this monograph. All are listed in the Handbook of Food Additives (383) as GRAS except helium; none are described in Food Chemicals Codex (359). From the Handbook, the following notations are found for the four gases listed:

Butane: GRAS as a miscellaneous and/or general purpose food additive under Section 121.101.

Nitrogen: GRAS as a miscellaneous and/or general purpose food additive under Section 121.101.

Cleared by MID to exclude oxygen from sealed containers, in an amount sufficient for the purpose.

Nitrous Oxide: GRAS as a propellant for certain dairy and vegetable-fat toppings in pressurized containers under Section 121.101.

Cleared for use in combination with octafluorocyclobutane as a propellant and aerating agent for foamed or sprayed food products, except for those standardized foods that do not provide for such use, under Section 121.1065 (Octafluorocyclobutane).

Cleared for use in combination with chloropentafluoroethane as a propellant and aerating agent for foamed or sprayed foods under Section 121.1180 (Chloropentafluoroethane) (June 10, 1965).

Propane: GRAS as a miscellaneous and/or general purpose food additive under Section 121.101.

Cleared for use in combination with octafluorocyclobutane as a propellant and aerating agent for foamed or sprayed food products, except for those standardized foods that do not provide for such use, under Section 121.1065. (Octafluorocyclobutane).

Cleared for use in combination with chloropentafluoroethane as a propellant and aerating agent for foamed or sprayed food products under Section 121.1181 (Chloropentafluoroethane).

There are two forms of butane: n-butane, which is that form used in this monograph; and isobutane which is not listed in the Handbook of Food

Additives. Both compounds occur in natural gas and crude petroleum, from which the butane is secured during the refinery cracking process. Butane is most generally known as a fuel, and in its liquified form reaches sales volumes of billions of gallons annually. Other uses for butane include it as a refrigerant and as a solvent used in the petroleum industry. Practically no literature has been found of biological studies with butane; its toxicity appears to be very low. Human exposure to 1% butane gas (10,000 ppm) for 10 minutes produces some drousiness but no other evidence of biological effects. The odor of the gas is slight, and not detectable below concentrations of 0.5% (5000 ppm). It is used in a controlled combustion technique for removing or displacing the oxygen during processing, packaging or storage of vegetable fats and oils, coffee, wine and citrus products.

Few studies on helium may be found related to biological effects or toxicity. Helium is the lightest element in the group of gases commonly referred to as the "rare", "noble" or "inert" gases; no one of these designations is entirely correct. More recently, the gases helium, neon, argon, krypton, xenon and radon have been known as the "helium-group" gases. These gases are widely distributed throughout the known universe; in the earth's crust most are contained in igneous rocks, from which they have been gradually released into the atmosphere. Most of the atmospheric helium today probably arose from the radioactive decay of the heavier elements releasing alpha particles which are helium atoms stripped of their two electrons. The principal commercial source of helium is natural gas, which in some wells may contain up to 8% helium.

Liquid helium is the coldest substance available in the field of cryogenics, with temperatures as low as a fractional part of 1 degree K achieved; commercial quantities are now available and liquid helium has largely replaced liquid hydrogen in cryogenics. Other uses for helium are in inert gas-shielded arc welding, as an inert environment for growth of semiconducting devices, and in gas lasers. Helium-filled ballons are used in upper atmosphere studies; liquid helium is found in rocketry and experimental nuclear rocket engines. Numerous other nuclear applications of helium may be listed; mixtures of helium and oxygen have been used in deep-sea diving to prevent nitrogen narcosis, and artificial atmospheres containing helium are used in medical therapy and anesthesia.

Schreiner (980) reported in 1968 the "general biological effects of the helium-xenon series of elements", indicating that the so-called "inert" gases are capable of showing biological activity, at sufficiently high concentrations. However, helium appears to be the least effective member of the group; men exposed to helium at 20 atmospheres pressure for 48 hours show no performance decrement. In monkeys and mice, convulsions occur at helium pressures of 45-47 atmospheres and 75-102 respectively. Helium-group gases are not essential tc life, produce physiological effects by modification of the gaseous environment, and show pharmacological effects by modification of cellular functions through interactions at the molecular level.

Nitrogen comprises almost four-fifths of the earth's atmosphere and, combined in various organic compounds such as proteins, is found in all

living cells. It is a colorless, odorless, diatomic gas, discovered in about 1772; the name means "nitre-forming" from the Greek. The Frenchman, Lavoisier, called it "azote" (without life) and the root "az" designates the many compounds containing nitrogen, such as azides, azines and azoles. At ordinary temperatures, nitrogen is relatively inert; at higher temperatures it reacts to form many compounds. Huge deposits of nitrates, potassium and sodium are found in natural sites in the East Indies, Bengal and Ceylon, Chile and Bolivia. High purity nitrogen is prepared in air-separation plants and is commercially available as compressed gas or liquid. The greatest use of nitrogen is in the synthesis of ammonia; other uses include numerous polymerization process, regeneration of catalysts in petroleum refining, glass manufacturing (as an inert atmosphere).

In the food industry, nitrogen is used to replace oxygen and thus prevent deterioration. Mold growth and insect infestation is also prevented by a nitrogen atmosphere in stored foods. Coffee, particularly the "instant" kind, is roasted in a nitrogen atmosphere, and ground coffee is canned in nitrogen. Margarines are whipped in a nitrogen atmosphere; leafy vegetables and similar perishables may be stored without refrigeration in nitrogen plus carbon dioxide. Meat packaged in plastic film may be pretreated with nitrogen to preserve an attractive appearance for long periods of time.

Nitrous oxide (dinitrogen monoxide or laughing gas) was discovered about the same time as nitrogen; it is a colorless gas with a slightly sweetish odor and taste. It has been used as a general anesthetic since 1844. The amount of nitrous oxide required for anesthesia, when mixed only with air, is so great that partial asphyxia occurs from oxygen insufficiency. Anesthesia can be induced by a mixture of 85% nitrous oxide and 15% oxygen inhaled for several minutes; the anesthesia is then maintained by 80% nitrous oxide and 20% oxygen.

Half a century ago, Davidson (245) performed studies of intoxication; nitrous oxide was administered to a healthy male volunteer in various concentrations and the general effects observed. Up to 30% nitrous oxide produced "lightness in the head", mild intoxication with paresthesia and some diminution in mental efficiency. At 30% concentration, slight muscular incoordination and lack of attention were observed; at 70-75% nitrous oxide, unconsciousness occurred suddenly, preceded by unintelligible speech and loss of sight before hearing and touch. The approximate times of onset of the various symptoms resulting from short inhalations of pure nitrous oxide and longer inhalations of various concentrations are shown in the following table:

TABLE 1

	CONCENTRATION OF NITROUS OXIDE				
	100 per cent	75 per cent	70 per cent	60 per cent	50 per cent
Commencing intoxication	7″–10″	30″	35″	45″	60″
Marked intoxication	20″	70″	90″	120″	150″
Talkativeness	30″	90″	90″–120″	150″	180″
Noisy talkativeness	40″	2′	3′ –3½′	4′	5′–6′
Aggressive and emotional	55″	3′	3½′–4′	4½′	6′–7′
Incoördination and unintelligible speech	60″	3½′	4′ –5′	5½′–6′	7′–8′*
Unconsciousness	60″–65″	4′–4½′	5′ –6′		

* Incoördination only.

Davidson studied reaction times, muscular effects, coordination in writing and typewriting, influence on memory, and estimates of elapsed time as influenced by inhalation of nitrous oxide.

Simpler (1022) has recently (1973) described the uses of nitrous oxide in the food and aerosol industries. He notes that a commercial source of the gas is by thermal decomposition of ammonium nitrate. The following information is taken directly from Simpler's report as a summary of the uses of nitrous oxide in foods and aerosols:

> "Certain of the properties of nitrous oxide--such as high solubility, chemical stability, its faintly sweet taste, and its acceptability in foods--lend themselves ideally to its use as a propellant in aerosol packaging. For many years nitrous oxide has been used as the propellant and whipping agent for pressurized whipped cream and, more recently, for vegetable-based whipped toppings. In such products, the can is filled and the cap and valve crimped in place. Then the valve is opened and the can charged with N_2O, under vigorous agitation to facilitate absorption of the gas. In use, the gas pressure forces the product-gas mixture out of the valve. On emergence into the reduced pressure of the atmosphere, from a container pressure of perhaps 100 pounds per square inch gauge pressure, the N_2O is released from solution, resulting in the foaming, or "whipping," of the product.
>
> Use in Other Preparations -- Much work has been in progress in recent years on the development of other food aerosols. Such proposed products include canape spreads, salad dressings and mayonnaise, and new whipped toppings and syrups. In some cases, such as in barbecue-sauce aerosol, a spray pattern is called for instead of an aerated stream. Nitrous oxide used as a propellant can also produce this spray effect with different valving arrangements.
>
> The same properties that make nitrous oxide so suitable for use in aerosol packaging make this gas an ideal medium for the aeration of fluffing of such food products as fluffy marshmallow, ice cream, and peanut butter.
>
> A significant portion of the food packaging field has found the use of flexible packaging as a lightweight, easy-to-ship, easy-to-use means of packaging food items. The applications in the field are varied and many, and include items such as dehydrated potatoes, sliced cheese, and "boil-in-the-bag" items. Many of these items are packaged in an atmosphere of inert gas as a means of excluding oxygen, which would cause the rapid deterioration of the product and loss in color, flavor, odor, and vitamin content.
>
> Employment as "Tracer Gas" -- Leakage in a flexible package is extremely difficult to detect by conventional methods. Presently under development is a method utilizing N_2O as a tracer

gas. Nitrous oxide is present only as a trace (0.00005 per cent by volume) in the atmosphere. It is therefore unique among the gases commonly used for leak detection and can be readily or easily traced. Basically, the system involves the mixing of a small amount of N_2O as part of the packaging gas; any leakage of the nitrous oxide from the flexible package would be detected by an infrared apparatus, which is capable of finding a few parts of N_2O in a million parts of background gas. This system, which is adaptable to a continuous processing and testing line, is nondestructive to the package and is therefore economical to use.

Recently this gas has found an interesting application as a tracer gas in the location of leaks in natural gas transmission lines. Very low concentrations of the gas are dissolved in the water used for hydrostatic pressure testing of the pipes. In the event of a leak, the nitrous oxide-laden water escapes from the pipe, the liberated nitrous oxide making its way to the surface of the ground where its presence in trace quantities can be determined by infrared sensing equipment.

Use in Frozen Food Production -- In the production of frozen foods, it is recognized that the more rapid the freezing rate, the better the quality of the product. With faster freezing, crystal size is smaller. This and other related factors minimize the cell damage and so-called extracellular freezing which, in the conventional slow-freezing processes, result in mushiness and drainage, with accompanying losses in flavor and appearance.

The most effective means of withdrawing heat from a product is by immersion of the product directly into a liquid refrigerant or a refrigerated medium. The physical characteristics of N_2O lend themselves well to this freezing technique. Its boiling point is sufficiently low for rapid freezing, and its relatively high heat capacity contributes to the efficiency of the process.

Under development is a freezing process involving the immersion of a food product directly into a bath of liquid N_2O at a temperature of -127.5° F. With this process, freezing rate is reduced from a period of hours, required with conventional practices, to a matter of minutes. Improvement in quality of such products as frozen strawberries and cherries is dramatic. Obvious improvement in quality can also be noted in such products as shrimp and other seafood, meats, and even frozen baked goods. A number of new products have been made possible by this new type of ultra-low-temperature freezing.

There are certain beverages which could benefit from effervescence, but in which acid formation would be detrimental from a flavor standpoint, or perhaps from the effect of the acid on the ingredients or on the container. Nitrous oxide

is being investigated as a means of "charging" these beverages. While it can theoretically be considered the anhydride of hyponitrous acid, this point is purely academic, since hyponitrous acid cannot exist in water and simply dissociates to water and N_2O. Therefore, a solution of N_2O in water brings about no change in pH.

Chemists and technicians are constantly studying new uses of nitrous oxide. In commercial use, "laughing gas" is becoming a "wonder gas" in the number of its contributions to better living in this modern age."

Blodgett and Webster (109) reported in 1959 on nitrous oxide and Freon C318 as propellants for aerosol food products. At that time, only nitrous oxide and carbon dioxide were used for edible sprays or foams, and nitrogen for edible nonaerated liquids. Freon C318 was being recommended for use in combination with nitrous oxide especially as a propellant for whipped cream.

Propane is a constituent of natural gas and petroleum from which it is secured by fractionation. It is widely used as a fuel, and is a source, by cracking, of ethylene and propylene. Propane is also used as a refrigerant and a selective solvent in oil refining and in extraction of oils from agricultural products. As was the case in butane, it is used in a controlled combustion technique for removing and displacing oxygen in the processing, storage and packaging of citrus products, vegetable fats and oils, wine and coffee.

Little, or no information, relating propane to foods has been found except that noted in the foregoing paragraph, and that extracted from the Handbook of Food Additives, indicating the combination with other compounds as a propellant and aerating agent.

Stoughton and Lamson (1073) experimented with mice to test the anesthetic properties of butanes and pentanes. They reported that n-butane in concentrations of 2.7-31% by volume inhaled for 2 hours was lethal to about half the animals tested. Lower concentrations, 13-22% butane, produced light anesthesia within 25 minutes (13%) or 1 minute (22%). The animals surviving the 2-hour treatment apparently recovered normally within several minutes.

In a recent report prepared for NASA, Hamilton et al. (453) described the results of experiments with mice and rats exposed for up to 6 months to a helium (80%) - oxygen (20%) atmosphere. Parent and two successive generations were raised in the helium atmosphere. Biochemical and histochemical tests showed no statistically significant differences between experimental and control animals.

Parbrook (837) exposed rats to several atmospheres for a week: air; 25% nitrous oxide, 21% oxygen, 54% nitrogen; and 60% nitrous oxide, 21% oxygen, 19% nitrogen. The higher nitrous oxide concentration (60%) lowered the white cell count significantly, compared to 25% nitrous oxide or air,

and several animals became ill with leucopenia, and one died. Wounded versus non-wounded animals showed a greater depression of the white cell count in those with wounds; animals with counts below 2500/mm3 became ill or died. Mouse studies gave similar results. The practice of post-operative treatment in humans with nitrous oxide to relieve pain was considered ill-advised beyond a 48-hour period, due to conditions of trauma and infection with a lowered resistance in the patient by the reduction of white cells.

Of the gases considered in this monograph, only nitrous oxide has been widely used in anesthesia, but an early study (1926) by Brown and Henderson (143) reported experimental exposure of cats to a propane atmosphere (60% propane, 40% oxygen). Anesthesia was obtained within about an hour. Higher concentrations of propane, varying from 82-93% were studied.

An early report (1927) by Henderson (477) discussed the toxicities of various anesthetics: chloroform, ether, nitrous oxide, ethylene, acetylene and propylene. Nitrous oxide was described as the least toxic, but also the least effective anesthetic. The chief hazard in the use of nitrous oxide lies in the resulting anoxia due to the anesthetic being absorbed by the body cells to the exclusion of oxygen.

A more recent study of the potential toxic effects of nitrous oxide was reported by Parbrook (839) who notes that hypotension and hypoxia are hazards associated with the use of nitrous oxide and diffusion toxic effects due to diffusion of the gas into air pockets in the body (inner ear, bowel and pneumothorax) may be encountered.

Observations made by Bloch (107) during the induction of hypothermia in patients undergoing anesthesia indicated that nitrous oxide produced rapid increase in blood pressure and heart rate following termination of the anesthesia. Withdrawal of nitrous oxide from an anesthetic mixture with oxygen, halothane, methoxyflurane or ether, relieves the depression of blood pressure and heart beat, but also reduced the depth of anesthesia.

Green (429) performed experiments with rats, exposing the animals to atmospheres of 80% nitrous oxide, 20% oxygen, for a period of 6 days, and observed that the white cell count dropped to less than 10% of normal values. A mortality rate of 50% was seen between the fifth and sixth days of exposure to nitrous oxide; progressive hypoplasia in the bone marrow and a disappearance of cellular mitoses occurred. Decreased concentrations of the nitrous oxide to 60% also appeared to be toxic to rat bone marrow; reduction to 40 and 20% nitrous oxide also lowers white cell counts but the animals appeared healthy after 3 weeks of exposure. The inhibition of mitoses appears to be a major influence of nitrous oxide; treatment of leukemia, however, with nitrous oxide has produced questionable results.

The potential hazards of exposure to anesthetic gases are not restricted to the patients; operating theatre personnel also run some risk. Nikki et al. (804) in a recent investigation of ambient concentrations of halothane and nitrous oxide in operating theatres and recovery rooms, found inefficient ventilation and possibly hazardous quantities in the atmosphere,

particularly in view of the high abortion rate among operating personnel and the potential teratogenic effect of nitrous oxide shown in animal studies. Corbett (215) also questioned the safety of the operating theatre, particularly in gynecology; concentrations of nitrous oxide used in anesthesia have been shown to range from 1000 to 3000 ppm in the zone occupied by anesthesiologists, and may even reach concentrations of 9700 ppm. Bone marrow depression in humans, and teratogenic and embryotoxic effects shown in rat studies, would indicate that pregnant women particularly, should not be exposed to operating theatre environments.

Eastwood et al. (297) established the depressive effect of nitrous oxide on rat white cell count; the normal count of 13,000 was reduced by 6 days of exposure to 80% nitrous oxide, 20% oxygen, to 1130. Therapy by nitrous oxide was applied to two cases of patients with greatly elevated white cell counts. A 3-year-old girl with a count of 170,000 was treated with nitrous oxide and a drop to 21,500 occurred within 72 hours. After treatment was terminated, the white cell count remained low, but the child developed an intracranial hemorrhage and died. The second patient, a 13-year-old boy with a count of 181,000 underwent a series of hospitalizations during which nitrous oxide therapy greatly reduced the white cell count. However, he too died, after several months from gross hematuria and a high fever.

Teratogenesis by exposure to nitrous oxide in rats was reported by Shepard and Fink (1010). Pregnant rats were exposed to 50% nitrous oxide for several days during embryonic age of fetuses of 8 days: embryonic death and resorption, and skeletal abnormalities were observed. Of 105 live fetuses produced, 15 were dwarfs, 57 showed vertebral ossification and 47 sectioned fetuses showed 4 with major visceral malformations. Maternal hypooxygenation was discounted as a causitive agent in the teratogenic action of nitrous oxide; more likely, the effect is due to decreased oxygen utilization at a cellular level in the embryo. A single-day exposure to 70% nitrous oxide indicated a maximum effect of the gas on a 9-day-old rat embryo which corresponds to the human embryo during days 17 thru 21 in development. Caution is suggested in the use of nitrous oxide in pregnant humans, particularly since pregnancy at that early date could hardly be anticipated.

A non-medical use for nitrous oxide was described by Lynn et al. (704) recently; the drug was being used by various individuals for "turning on". Sources of the gas include aerosol cans or tanks used in whipped cream preparations, and various professionals (doctors, nurses, dentists and therapists). The authors conducted experiments with volunteers who were asked to inhale pure nitrous oxide; within 15-30 seconds, exhilarating "highs" were experienced lasting several minutes. Cognitive functioning tests did not reveal a significant difference between naive and sophisticated volunteers. Minimal adverse effects were observed, usually among "first-users". Transient nausea, dry throat, bloating, and heat pressure were experienced by some, as well as mild suspicionsness.

Mice raised after age 45-55 days in an atmosphere of 80% helium, 20% oxygen showed abnormal cardiac tissue mitochondria in studies performed by Hawkins (467) recently. Electron microscopy of cardiac tissue revealed

altered mitochondria, particularly those near the transverse sarcotubular apparatus. Frequently, both inner and outer mitochondrial membranes appeared to be ruptured. It was possible that the removal of nitrogen from the atmosphere in which the animals spent the first 45-55 days of life contributed to the mitochondrial effect. Hawkins (468) also reported a year later on the effects of helium on the mouse skeletal muscle fibers. The experimental materials and methods were those used in the mitochondrial study. Diffuse myopathic lesions were seen in photomicrographic sections of muscle tissue; the lesions were fusiform, clumps beaded along the length of the muscle fiber. It was an effect similar to the appearance of a dystrophic myopathy.

A difference in the response to a nitrous oxide atmosphere (70% nitrous oxide, 20% oxygen, 10% nitrogen) by two strains of rats, Sprague-Dawley and Long-Evans, was reported by Green (430). White cell counts were more significantly reduced in the Long-Evans strain, and the DNA content was lower. Small effects on the RNA and DNA in Sprague-Dawley animals were seen, but a marked alteration of the RNA/DNA ratio occurred in the Long-Evans strain.

The effects of nitrous oxide on lipid metabolism in guinea pig and in man were described by Kosmider and Misiewicz (619). Male guinea pigs, 50 controls and 30 experimentals were used experimentally. The test animals were exposed 8 hours daily for 180 days to an atmosphere containing 1 ppm nitrous oxide. Determinations of total serum free and esterified cholesterol and total lipids showed significant increases in the test animals compared to controls. Liver cholesterol levels, however, were significantly lower in the test animals, but the total lipids did not differ essentially from controls.

Epidemiological studies of 70 male workers chronically exposed to nitrous oxide in a chemical factory (concentration nitrous oxide ranged between 0.4 and 2.7 ppm), daily during working hours for from 4 to 6 years, revealed increased total lipids compared to non-exposed controls. The alpha lipid fractions were lower and the beta-gamma fractions increased over control values. The chief difference between guinea pig and man, exposed chronically to nitrous oxide, was in the increased cholesterol levels in the guinea pig and the decreased cholesterol in man.

BUTANE

Chemical Information

I. Nomenclature

A. Common Name

Butane

B. Chemical Names

Butane
n-Butane
Di-ethyl

C. Trade Names

None found

D. Chemical Abstracts Registry Number

000106-97-8

II. Empirical Formula

C_4H_{10}

III. Structural Formula

$CH_3CH_2CH_2CH_3$

IV. Molecular Weight

58.12

V. Specifications

From NAS/NRC Questionnaire (Pure Grade Propellants):

Composition, mol %	
Isobutane	max 1.0
Normal butane	min 99.0
Pentanes	trace
Total Saturates	min 99.9
Vapor Pressure, psig at 70 degrees F	17 ± 2

V. Specifications Con't.

Sulfur, ppm	max 5
Residue, g/100 ml	max 0.0003
Acidity of Residue	neutral
Odor	nil

VI. Description

A. General Characteristics

Flammable gas, colorless, odorless.

B. Physical Properties

Flash point - 138 degrees, b.p. - 0.50 degrees, dgas 2.046 (air = 1). Soluble in H_2O at 17 degrees and 770 mm, 0.15 vol. of the gas in 1 vol. H_2O; 18 vol gas in 1 vol. alcohol.

C. Stability

No Information Available

VII. Analytical Methods

No Information Available

VIII. Occurrence

Butane is a component of natural gas and of petroleum, from which it is secured as a refinery cracking product.

HELIUM

Chemical Information

I. Nomenclature

A. Common Name

Helium

B. Chemical Name

Helium

C. Trade Name

None found

D. Chemical Abstracts Registry Number

007440-59-7

II. Empirical Formula

He

III. Structural Formula

Not applicable

IV. Atomic Weight

4.0026

V. Specifications

No Information Available

VI. Description

A. General Characteristics

Colorless, odorless, nonflammable, inert gas.

B. Physical Properties

Density 0.1368 (air = 1); solidifies at -271.9 degrees at 26 atmospheres. Very slightly soluble H_2O, 0.97 ml/100 ml at 0 degrees, 1.08

ml/100 ml at 50 degrees.

density (32°F, 1 atm), lb/ft^3	0.01114
boiling point (1 atm), 0°F	-452.0
melting point (1 atm), °F	26 atm -458.0
vapor density at bp, lb/ft3	0.999
liquid density at bp, lb/ft3	7.803
vapor pressure (solid at mp), mm Hg	
heat of vaporization at bp, Btu lb	10.3
heat of fusion at mp, Btu/lb	1.8
Cp(59°F. 1 atm), Btu/(lb)(0°F)	-292°F 1.25
Cp/C, (59-68°F, 1 atm), Btu/(lb)(0°F)	-292°F 1.66
critical temperature, 0°F	-450.2
critical pressure, psia	33.2

C. Stability

No Information Available

VII. Analytical Methods

No Information Available

VIII. Occurrence

Found in natural gas from which it is extractd on a commercial scale; produced in the decay of radioactive elements; also produced by bombardment of beryllium, lithium and other light elements with cosmic rays, x-rays and high-speed protons and denterons; such bombardments take place in nature and give a constant supply.

NITROGEN

Chemical Information

I. Nomenclature

A. Common Name

Nitrogen

B. Chemical Name

Nitrogen

C. Trade Name

None found

D. Chemical Abstracts Registry Number

007727-37-9

II. Empirical Formula

N (N_2 as diatomic molecule)

III. Structural Formula

Not Applicable

IV. Atomic Weight

14.008

V. Specifications

See following page.

TABLE 1

Limiting Characteristics	GRADES													
	A	B	C	D	E	F	G	H	J	K	L	M	N	P
Nitrogen Min., % (v/v) (Note)	97	99.0	99.5	99.5	99.5	99.9	99.95	99.99	99.99	99.995	99.998	99.999	99.9985	99.999
Water	None condensed			26.3	26.3	32.0	26.3	11.4	3.5	16.2	3.5	1.5	1.5	1.5
Dewpoint, °F				—63	—63	—60	—63	—75	—90	—70	—90	—100	—100	—100
Hydrocarbons, condensed									0.1 (wt/wt) see 8.3					
Total Hydrocarbons, (as methane)					58.3			5	3				1	
Oxygen			0.5%	0.5%	0.5%	0.1%	500	50	50	20	10	5	1	1
Hydrogen													1	
Argon, Neon, Helium													5	
Carbon Monoxide													1	
U.S.P.		Yes												
Permanent Particulates					Type II Less than 1.0 mg/1 See 5.9 Requires filtering See 5.9.1			Type II Less than 1.0 mg/1 See 5.9	Type II Less than 1.0 mg/1 See 5.9 Requires filtering See 5.9.1 Particulate population See 5.9					

NOTE: Unless shown otherwise % N_2 includes trace quantities of neon and helium and small amounts of argon.

VI. Description

A. General Characteristics

Odorless gas.

B. Physical Properties

Sparingly soluble H_2O, 2.4 vol. N in 100 vol. H_2O at 0°, 1.6 vol. at 20°. Soluble in liquid ammonia and alcohol. Boiling point - 195.8^o, solidifies to snow-white mass with melting point - 209.8^o. Combines with oxygen to form nitric oxide and with hydrogen to form ammonia on sparking. Combines directly with lithium, and at red heat with calcium, strontium and barium to form nitrides. Forms cyanides when heated with carbon in presence of alkalies or barium oxide.

C. Stability

No Information Available

VII. Analytical Methods

Bowes (125) has described a method in considerable detail for measuring N_2O, N and O_2 in the blood by the use of gas chromatography to analyze the gas contents of a small bubble which has been equilibrated with those gases dissolved in the blood. See Documents for details. Bennett (75) has described a gas chromatographic analysis of gas mixtures (including N_2).

VIII. Occurrence

Comprises 77.5% by weight (78.06% by volume) of the atmosphere. Frequently found in volcanic or mine gases and gases from springs; also in occluded gases of minerals and rocks. Constituent of all living protoplasm. Prepared industrially by fractional distillation of liquid air.

NITROUS OXIDE

Chemical Information

I. Nomenclature

A. Common Names

1. Nitrous oxide
2. Dinitrogen monoxide
3. Laughing gas
4. Hyponitrous acid anhydride
5. Factitious air

B. Chemical Name

Nitrous oxide

C. Trade Name

None found

D. Chemical Abstracts Registry Number

010024-97-2

II. Empirical Formula

N_2O

III. Structural Formula

Not Applicable

IV. Molecular Weight

44.02

V. Specifications

RAW MATERIAL SPECIFICATIONS

1. Description — Nitrous oxide, N_2O, in liquid form, meeting USP requirements.

2. Approved Supplier

V. Specifications Con't.

3.	Packaging	Delivered in liquid form to storage containers on customer's property.
4.	Appearance	Colorless gas at room temperature and pressure.
5.	Purity (Guaranteed)	99.5% Minimum
6.	Impurities (by volume)	

Nitrogen	0.5%	Maximum
Carbon dioxide	1 ppm	Maximum
Higher oxides of nitrogen	0.5 ppm	Maximum
Sulfur oxides	0.5 ppm	Maximum

VI. Description

A. General Characteristics

Colorless gas. Slightly sweetish odor and taste. Stable and inert chemically at room temperatures. Supports combustion.

B. Physical Properties

Dissociates above 300 degrees becoming strong oxidizing agent. Melting point - 90.81°; boiling point (at 760 mm) - 88.46°. One liter at stp weighs 1.977 g. Soluble in H_2O, 1 liter gas/1.5 liters H_2O, at 20° and 1 atmosphere; freely soluble in sulfuric acid. Soluble in alcohol, ether, oils.

C. Stability

Stable and chemically inert at room temperatures.

VII. Analytical Methods

Davies (247) has described a gas chromatography technique for the quantitative analysis of blood gases. See Document Section for details. See also analytical methods under nitrogen, Bowes (125). Kennedy (575) described a micro method for the determination of N_2O in blood. See also Bennett (75) under Nitrogen, Analytical Methods.

VIII. Occurrence

Constituent of earth's atmosphere, about 0.00005% by volume. Comercially prepared by thermal decomposition of ammonium nitrate.

$$NH_4NO_3 \xrightarrow{170^\circ-260^\circ} N_2O + 2H_2O$$

Contaminants usually present in commercial N_2O are: N_2, NO_2, NO, O_2 and CO_2.

PROPANE

Chemical Information

I. Nomenclature

A. Common Name

Propane

B. Chemical Names

Propane
Dimethylmethane
Propyl hydride

C. Trade Name

None found

D. Chemical Abstracts Registry Number

000074-98-6

II. Empirical Formula

C_3H_8

III. Structural Formula

$CH_3CH_2CH_3$

IV. Molecular Weight

44.09

V. Specifications

No Information Available

VI. Description

A. General Characteristics

Odorless when pure. Burns with luminous, smoky flame. Heavier than air (2.0200g/liter at stp).

B. Physical Properties

Liquefies at -42°, solid at -187.7°, boiling point at one atmosphere, -42.1°. Critical temperature 96.81°, critical pressure 42.01 atmosphere. Increasingly soluble in H_2O, absolute alcohol, ether, chloroform, benzene and turpentine. Forms solid hydrate with H_2O at low temperatures.

C. Stability

No Information Available

VII. Analytical Methods

No Information Available

VIII. Occurrence

Constituent of natural gas and of crude petroleum from which it is obtained by fractional distillation under pressure.

Biological Data

I. Acute Toxicity

Mouse (butane)

An early (1936) experiment by Stoughton and Lamson (1073) with butanes and pentanes administered to mice to observe the anesthetic properties of these gases indicated that n-butane was lethal in concentrations of 27-31% by volume. Five mice at a time were placed in a 20-liter bottle for 2 hours in atmospheres with different concentrations of the gas studied. Butane produced light anesthesia within 25 minutes at 13% and within 1 minute at 22%. Concentration of 27% butane produced loss of posture in the mice within 4 minutes, and by 2 hours, 40% of the animals were dead; at 31% butane, loss of posture occurred within 3 minutes, and 60% were dead after 2 hours. Ten mice were used at each concentration, and the LD_{50} lay between 27 and 31% butane. Those animals surviving the 2-hour exposure recovered quickly, usually within several minutes, and appeared normal.

II. Short-Term Studies

Mouse (helium)

An elaborate, detailed, study for NASA by Hamilton et al. (453) presents the results of experiments with rats and mice maintained for up to 6 months in a carefully controlled atmosphere of 80% helium and 20% oxygen, with other environmental parameters kept constant. Controls lived in an environment differing only in substitution of nitrogen for helium in the atmosphere.

Forty parent generation mice of the inbred strain, Manor Swiss MF-1 were employed in the mouse experiments; half of the animals were placed in a helium-oxygen environment and half in a nitrogen-oxygen chamber. Offspring of the original parents, or the F1 generation were randomly culled to 40 individuals who were then mated, to produce a second generation (F2). During the course of the experiment, a total of 160 F1 mice and 60 F2 mice were called from the helium-oxygen chamber, and 169 F1 and 98F2 mice were called from the nitrogen-oxygen chamber. Six rats were placed in each chamber at the beginning of the experiment; they did not breed and were used for oxygen consumption studies at the end of the 6-month exposure.

Helium adapted animals consumed no more oxygen than the controls and the growth rates were the same for both group even though the helium treated mice consumed more food and water.

Parent and two successive generations of mice were raised in helium-oxygen. Biochemical analyses including blood indices; electrophoretically separated tissue protein patterns from liver, skeletal muscle and cardiac

muscle; quantitative determinations of LDH, MDH and G6PDH (lactate, malate and glucose-6-phosphate dehydrogenases) from the same tissues; serum insulin; and semi-quantitative histochemical estimates of liver glycogen, were performed and showed no statistically significant differences between experimental and control animals. Liver nucleotides and redox-coenzymes determinations also reveal no significant differences.

Relative weights of liver, heart, kidney and diaphragm were the same in both groups; histopathological findings showed no remarkable difference. It was concluded that prolonged exposure to helium-oxygen, relative to air, produced no detectable changes in several key subcellular factors which would indicate serious metabolic disturbances.

This report consists of 86 pages and should be referred to in the Documents section for details.

Rat (nitrous oxide)

Parbrook (837) subjected Norwegian hooded rats to several atmospheric compositions over a period of several weeks to determine the effects of prolonged nitrous oxide treatment. Three groups of 12 each were exposed, each to a different atmosphere, of either air, 25% N_2O + 21% O_2 + 54% N_2, or 60% N_2O + 21% O_2 and 19% N_2. Two animals from each group developed inflammation in the tail, from which blood samples were removed for white blood cell counts. The results of the three groups in the three atmospheres with respect to white cell counts are shown in the following figure:

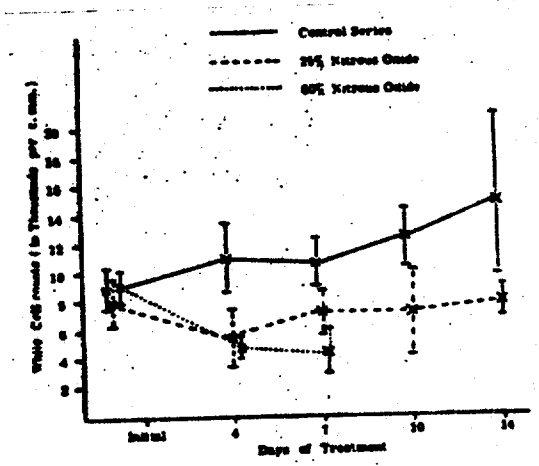

FIG. 1

The graph indicates the mean white cell counts and 95 per cent confidence limits for three comparable groups, each of 10 rats, exposed to air or to one of two concentrations of nitrous oxide. The leucocytosis in the control group was attributed to tail trauma from repeated taking of blood samples.

The rats treated with 60% N_2O had lower white cell counts than did controls on the 4th day of treatment, and by the 7th day, the counts were significantly lower than those of the 25% N_2O group, which were also lower than controls. However, the 60% group on the 7th day had 2 animals ill with leucopenia, and a third that died from marked leukopenia; it appeared inadvisable to continue with this group and the animals were destroyed.

Nitrous oxide is frequently given for postoperative pain, and Parbrook undertook an experiment with 3 groups of 8 rats each, treated for only 4 days. Some animals were given incised wounds; the results, shown in the following table indicate a rise in white cell count in control "wounded and "nonwounded", and depression of white cell counts in both 25% and 60% N_2O. Those animals with wounds showed the greater depression of white cell counts and a higher morbidity. Animals with greater than 2500/mm3 white cells remained in satisfactory health; those animals with lower counts appeared ill or died.

TABLE I

Mean changes in white cell counts (per cu.mm) after 4 days of inhaling air or nitrous oxide in 25 or 60 per cent concentration.

	Controls (air)			25 per cent nitrous oxide			60 per cent nitrous oxide			Analysis of variance		
	N	$\bar{x}$	SE	N	$\bar{x}$	SE	N	$\bar{x}$	SE	F	d.f.	P
Rats without wounds	10	+2,200†	969	10	−2,570*	984	10	−4,125†	672	13.8	2:27	<0.0005
Rats with wounds	7	+5,529*	1,869	8	−5,494†	882	7	−6,579†	638	29.3	2:19	<0.0005

$\bar{x}$ = mean of changes in white cell counts. N = number of animals in each group.
SE = standard error. * Probability, P<0.05. † P<0.01.
Comparing all treated non-wounded with treated wounded animals, the increased depression of the count in the latter is significant (t_{13}=3.26; P<0.01).

The duration of exposure to 25% or 60% N_2O of wounded animals influenced the white cell counts as shown in the following table for exposures of 1, 2, 3 and 4 days.

TABLE II

Mean changes in white cell counts (per cu.mm) after 1, 2, 3 and 4 days in rats with wounds.

Duration of treatment (days)	Controls (air)			25 per cent nitrous oxide			60 per cent nitrous oxide			Analysis of variance		
	N	$\bar{x}$	SE	N	$\bar{x}$	SE	N	$\bar{x}$	SE	F	d.f.	P
1	6	+1,342	1,667	6	−2,675*	922	6	−2,783	1,334	3.1	2:15	<0.1
2	14	+ 929	719	14	−2,004†	569	13	−3,369†	867	9.2	2:38	<0.001
3	6	+2,325	1,502	6	−2,933*	1,047	6	−4,092*	1,569	7.8	2:15	<0.01
4	7	+5,529*	1,869	8	−5,494†	882	7	−6,579†	638	29.3	2:19	<0.0005

$\bar{x}$ = mean of changes in white cell counts. N = number of animals in each group.
SE = standard error. * Probability, P<0.05. † P<0.01.
The rats at each day are different sets except for those which were assessed at 1 and 3 days after the start of the experiment. The counts were made 2 hours after treatment in the "1 day" results and 18 to 20 hours after removal from the atmospheres in the results of the 2, 3 and 4 days.
Duration of treatment. Analysis of trend, in the 2-, 3- and 4-day results:
Controls: the increasing leucocytosis is significant; t_{11}=2.77; P<0.02.
25 per cent nitrous oxide: the increasing leucopenia is significant; t_{13}=3.38; P<0.01.
60 per cent nitrous oxide: the increasing leucopenia is significant; t_{11}=3.53; P<0.01.

Similar studies with mice gave similar results with 25% and 40% N_2O. Differential counts in rats and mice in exposures of 3 days of 25% and 60% N_2O showed a highly significant reduction of the polymorphonuclear percentage. Continuous nitrous oxide therapy in humans appeared to the

author undesirable beyond a period of 48 hours; a risk of inhibition of leukocytosis by N_2O could not be tolerated in view of postoperative trauma and infection, lowering the patient's resistance.

III. Long-Term Studies

None Found

IV. Special Studies

Anesthesiology

Brown and Henderson (143) in 1926 described the results of administering propane, butylene or propylene to cats as an anesthetic agent. Two cats, each weighing about 3 kg, were given propane in several concentrations over several hours time. In the first experiment, a mixture of 60% propane and 40% O_2 was given to begin; within 15 minutes, the propane concentration fell to 43% and the mixture was altered to 62% propane. Within an hour, anesthesia appeared to be complete; the oxygen concentration was slowly increased, reducing propane to 43%, but 25 minutes later, the propane was again increased, to 53%, and still later, to 65%. During the last hour of the experiment, complete anesthesia was maintained. The second experiment was similarly conducted, but with higher propane concentration varying from about 82 to 93%.

In an early report (1927), Henderson (477) discussed the toxicity of several anesthetics in use at that time: chloroform, ether, N_2O, ethylene, acetylene and propylene. Of N_2O, Henderson states that "it seems to be inherently the least toxic of any of our anaesthetics, but is undoubtedly the least efficient". It is such a weak anaesthetic that it must be combined with a certain amount of anoxaemia or other depressants of the central nervous system such as morphine or scopolamine. Though nitrous oxide appears not at all toxic in itself, it is possible that it is absorbed by the body cells to the exclusion of sufficient oxygen; anaesthetic deaths with nitrous oxide may have been the consequence of overloaded heart muscle working to supply sufficient oxygen to the body.

Parbrook (839), more recently (1971), observed that although N_2O is one of the safest anaesthics with minimal effects on metabolism, there are several potential toxic effects associated with its clinical use. Hypotension and hypoxia are hazards in the use of N_2O, as are diffusion toxic effects due to diffusion of nitrous oxide into pockets of air in the body (inner ear, bowel, pneumothorax, etc.). It has not been demonstrated in humans that N_2O is teratogenic, but the real danger exists during the first few weeks of pregnancy, before the patient realizes that she is pregnant.

Observations made on patients during the induction of hypothermia under general anaesthesia led to an investigation by Bloch (107) to determine the systemic effects of N_2O. Nitrous oxide, oxygen, halothane, methoxyflurane or ether were used in anaesthesia; when the anaesthesia, but not the cooling (patient lies on a mattress-type surface-cooling unit), is terminated, blood pressure and heat rate rise rapidly, often within 5 minutes. Varying the concentrations and mix of the anaesthetics, and observations made during both normal and hypothermic conditions indicated that changes in heart rate and blood pressure may occur as a result of anaesthesia with N_2O alone, irrespective of other anaesthetics used. Withdrawal of N_2O from anaesthetic mixes with oxygen together with halothane, methoxyflurane or ether, relieves the depression of blood pressure and heart rate, and is accompanied by reduction in the depth of anaesthesia.

The editorial comment introduces a report by Green (1964) concerning the toxicity of nitrous oxide. Green observes that up to the mid-nineteen fifties, N_2O was considered nontoxic. A number of studies indicated, however, that prolonged inhalation of N_2O led to bone marrow destruction, megaloblastic erythropoesis and granulocytic changes characteristic of pernicious anemia. Green exposed albino rats to an atmosphere of 80% N_2O and 20% O_2 for 6 days whereupon the white cell count dropped to less than 10% of normal values. Granulocytes disappear from the blood more rapidly than lymphocytes, and abnormal platelets are seen in the peripheral blood. A mortality rate of 50% is seen between the 5th and 6th days of exposure to the N_2O. Bone marrow in the rats shows progressive hypoplasia, with cellular mitoses disappearing. Sixty percent N_2O, 20% O_2 and 20% N_2 also appear toxic to rat bone marrow; 40% and 20% N_2O reduce white cell counts, but animals appear healthy after 3 weeks of exposure with white cell counts remaining over 50% of normal.

The inhibition of cell growth (mitosis) appears to be one of the most apparent influences of N_2O. Treatment of leukemia with N_2O has yielded questionable results; white cell counts are greatly reduced (from more than 100,000/mm^3 to about 15,000/mm^3), but progressive bone marrow destruction has been observed. The toxic properties of N_2O are not manifest during its use for less than 48 hours.

The potential hazards of anaesthetic gases are not restricted to the patient; personnel chronically exposed in operating theatre and recovery room also run risks, particularly in view of the high abortion rates among anaesthetic personnel. Nitrous oxide has been shown in animal studies to be haematotoxic and teratogenic in high concentrations.

Nikki et al. (804) recently made a survey of ambient concentrations of halothane and nitrous oxide in operating theatres and recovery rooms with and without gas scavenging systems. It was found that uneffective ventilation appeared to be common in many operating theatres, and recovery rooms too often were not located apart from operating theatres. A temporary scavenging system was employed in the studies; ambient halothane concentrations were reduced by more than 90%, and nitrous oxide concentrations by about 85% by the system. The findings of the survey are shown in the following tables:

TABLE 1

Concentrations of halothane and nitrous oxide in operating theatres.

Sample	Halothane (parts per million, v/v)				Nitrous oxide (parts per million, v/v)			
	Number	Mean	SD	Range	Number	Mean	SD	Range
General no scavenging	34	13.05	7.62	2.3 —30.7	28	890	664	90 – 2750
Individual no scavenging	44	14.09	12.48	2.4 —57.0	34	971	662	60 – 2500
All samples no scavenging	78	13.85	10.43	2.3 —57.0	62	929	659	60 – 2750
All samples with scavenging	27	0.85	0.95	< 0.01— 1.9	26	135	191	25 – 380

TABLE 2

Concentrations of halothane and nitrous oxide in recovery rooms.

Sample	Halothane (parts per million, v/v)				Nitrous oxide (parts per million, v/v)			
	Number	Mean	SD	Range	Number	Mean	SD	Range
Recovery rooms (2) located apart from operating theatres	18	2.31	0.85	< 0.01—3.6	18	120	99	< 5—430
Recovery room (1) close to operating theatres, (when no scavenging in op.th.)	14	3.38	1.78	0.8 —8.2	19	192	136	37—500

Gurney (443) described the use of N_2O in dentistry noting: the non-toxicity of the gas; the advantages of its use; use in combination with premedication; the disadvantages (must avoid a drift by the patient from hypoalgesia to analgesia); how it should be administered; the subjective symptoms (euphoria, lethargy, drousiness and temporary amnesia); dangers of withdrawal actions; and the recovery of the patient.

Corbett (215) has recently (1972) questioned the safety of various anesthetics used in gynecology, particularly the concentrations of anesthetics in the operating theatre. Concentrations of N_2O in the inhalational zone of anesthesiologists have been shown to range from 1000 to 3000 ppm, and may peak on occasion to 9700 ppm. No present limits have been established for exposure to most anesthetic agents, and through N_2O is considered an "inert gas", several deleterious effects from chronic exposure have been observed: bone marrow depression in humans, and teratogenic and embryotoxic effects in rats. It is suggested that, in the absence of evidence regarding the toxic effects of anesthetics, pregnant women employed in an operating room environment should stay out during pregnancy. Further studies of the possible carcinogenicity, teratogenicity and embryotoxicity of anesthetics, such as N_2O - should be pursued.

Therapy

Eastwood et al. (297) attempted therapeutic treatment of 2 patients with N_2O after first establishing the depressive effects in white rats of the white-cell count by N_2O. Thirty-two controls and 25 experimental albino rats were placed in chambers with constant temperature, humidity and gas concentrations. Controls breathed air; experimental rats breathed 80% N_2O plus 20% oxygen, which produced a state of intoxication resulting in slightly lowered food and water intake. The controls showed an average, normal white-cell count of 13,000. The mean count of the experimental animals exposed to N_2O dropped progressively to 1130 by the sixth day of exposure. Progressive hypoplasia of the bone marrow in experimentals led to cessation of cellular mitoses and reproduction.

Two case reports were given: a 3-year-old girl with an initial white-cell count of 60,000; and a 13-year-old boy with a white-cell count of 181,000. During therapy the girl's count rose to 170,000 upon which N_2O was administered with subsequent lowering of the count to 21,500 within 72 hours. For several days after N_2O therapy was discontinued, the count remained low, but the patient died, with evidence of intracranial hemorrhage. The boy underwent a series of hospitalizations with successful lowering of the white-cell count, but after several months developed gross hematuria and a high fever, and died.

The mechanism by which N_2O depresses bone-marrow may be related to the mechanisms of anesthesia.

Teratology

Similarities of rat and man with regard to the susceptibility of the bone marrow in each to N_2O suggests a special usefulness in using the rat as a mammalian species in experiments with N_2O. Shepard and Fink (1010) used pregnant Sprague-Dawley rats to observe the embryonic effects, exposing the animals under various conditions to different concentrations of N_2O. Exposure to 50% N_2O for 2 to 6 days at age 8 days of the embryo produced death and resorption and abnormalities of vertebrae and ribs. The tabular results are shown in the following table:

TABLE 26.1

Weight, length, sex ratio and incidence of malformations in rat fetuses from mothers placed in 50% N_2O and 21% O_2-N_2 on day 8 of pregnancy, and from 21% O_2-N_2 controls

Days in 50% N_2O	Implantations	Resorptions	Survivors	Average weight	Average length	Sex ratio*	Survivors with skeletal malformation	Other malformation
				g	mm			
0 (Control)	77 (6)†	1	76	3.7 ± 0.1‡	36.5 ± 0.2‡	39:37	2 (46)§	0 (29)§
2	36 (3)	7 (19%)	29 (81%)	3.2	34	11:18	15 (15)	0 (14)
4	88 (7)	22 (25%)	66 (75%)	2.8	31	22:44	42 (42)	4 (23)‖
6	23 (2)	13 (57%)	10 (43%)	2.7	33	2:8	(0)	0 (10)
Experimental totals	147 (12)	42 (28%)	105 (72%)	2.9 ± 0.1	31.8 ± 0.3	35:70	57 (57)	4 (47)

* Ration of male:female.

† The number of litters is in parentheses.

‡ Results given ± standard error.

§ The number of fetuses examined is in parentheses.

‖ Excluding one dwarf preserved intact for photography.

Of the 105 live fetuses from experimental litters, 57 were stained with alizarin, showing defects of vertebral ossification. Fifteen of the 105 were dwarfs; many types of skeletal abnormalities were encountered. Forty-seven fetuses were sectioned; 4 had major visceral malformations including hydronephrosis, cardiomegaly and hydrocephaly.

Other groups of animals were starved for 24 hours and treated for 24 hours with 70% N_2O or for 48 hours with 10% O_2. The results of these experiments are shown in the following table:

TABLE 26.2

Weight, sex ratio and incidence of defects in rat fetuses from control, 24-hr starved, 24-hr 70% nitrous oxide exposure and 48-hr 10% oxygen exposure

Treatment	Litters	Resorptions	No. of corpora lutea of pregnancy	Survivors				Skeletal findings			
				No. surviving	No. of fetuses per litter	Average weight ± S.E.	Sex ratio*	No. of fetuses examined	Fetuses with		
									Abnormal vertebrae or ribs	Extra ribs	Less than six sternal ossification centers
						g			%		
Controls											
None	22	8	288	256	11.6	3.75 ± 0.03	129:127	161	2	14	6
Day 19	2	0	22	19	9.5	2.1		19	5	5	100
Starved controls											
Day 7	7	3	94	84	12.0	3.57 ± 0.14	38:46	71	0	15	18†
Day 8	12	7	147	128	10.7	3.59 ± 0.10	50:78	80	5	19	9
Day 9	9	5	106	94	10.4	3.66 ± 0.09	46:48	94	0	20	7
Hypoxia 10% O_2											
Days 8 and 9	3	1	38	37	12.3	3.47 ± 0.05	21:16	37	8	5	19‡
70% Nitrous oxide											
Day 5	2	1	31	29	14.5	3.07 ± 0.10§	15:14	29	3	0	59†
Day 6	5	4	83	76	15.2	3.25 ± 0.10§	35:41	76	1	14	26†
Day 7	13	2	164	161	12.4	3.05 ± 0.07§	69:92	147	14†	26†	21†
Day 8	8	2	95	91	11.4	3.36 ± 0.09§	37:54	91	25†	36†	20†
Day 9	8	8	120	108	13.5	3.37 ± 0.12§	60:48	108	54†	7	58†
Day 10	8	4	93	101	12.6	3.70 ± 0.20	55:46	78	6†	1	6
Day 11	4	4	50	42	10.5		19:23	32	3	0	
90% Nitrous oxide											
Day 8	8	7	98	77	9.6	3.52 ± 0.05	38:37	76	12†	29	5

* Ratio of males to females.

† Difference between control and treated groups significant at the 1% level or less. Calculated by the χ^2 method.

‡ Difference between control and treated groups significant at the 5% level. Calculated by the χ^2 method.

§ Difference from untreated average weight significant at the 5% level using the t test.

Speculation by the authors concerning the mechanism of N_2O action in the pregnant rats discounted maternal hypooxygenation and suggested decreased oxygen utilization at the cellular level in the embryo, slowed cellular multiplication, cell death, or disruption of enzyme induction. The results of single-day exposure to 70% N_2O (Table 26.2) indicated a peak incidence of embryonic malformation on day 3 which corresponds to the human embryo development during days 17 through 21 of gestation. Any practical importance of the use of anesthetics to teratogenesis in the human requires further investigation.

Drug Abuse

A preliminary study of the non-medical use of nitrous oxide was reported by Lynn et al. (704) in 1971. The Mid-Michigan use of the agent by individuals for "turning on" was surveyed, and the results of cognitive functioning tests were reported. The chief source of the gas was from aerosol cans or tanks used in the preparation of whipped cream; various medical professionals (doctors, nurses, dentists and therapists) also provided the agent at parties. A pilot study, using ten volunteers who were asked to inhale pure N_2O, revealed exhilarating "highs" within 15-30 seconds after inhalation, the peak effects lasting 2-3 minutes. All described the experience as "pleasurable", with a state of well being lasting for minutes or hours after inhalation of N_2O. Subsequent use of the gas by the volunteers generally led to a "reverse tolerance" with more intense reactions. Cognitive functioning tests given at 5 and at 10 minute periods following inhalation by drug-naive and sophisticated volunteers showed little difference between the two groups. Adverse effects were minimal, chiefly among the naive, or first-users, and included some transient nausea, bloating, head pressure, dry throat and a mild suspiciousness. These defects were transient and a mild decrease in cognitive functioning during the peak effects usually lasted only 5 minutes.

Biochemical Aspects

I. Breakdown

No information found

II. Absorption-Distribution

No information found

III. Metabolism and Excretion

No information found

IV. Effects on Enzymes and Other Biological Parameters

Hawkins (467) reported the results of substituting an atmosphere of 80% helium, 20 oxygen for one of 70% nitrogen, 21% oxygen in A/Jax mice 45-55 days old. Ten animals were placed in the helium-oxygen atmosphere and ten controls in nitrogen-oxygen for an unspecified time. At the end of the experiment, the animals were anesthetized and still-living tissues removed from the top of the left ventricle of the heart. Tissue sections were prepared and stained and examined by electron microscopy. Mitochondria in cardiac tissue from control animals showed no changes; in test animals, the mitochondria, particularly those near the transverse sarcotubular apparatus, showed marked alteration, frequently with both inner and outer membranes appearing ruptured. The use of He in the experiment required the removal of nitrogen, and it was possible that removing nitrogen may have contributed to the mitochondrial effect.

The author (Hawkins (468)) also studied the effects of helium on mouse skeletal muscle fibers. The materials and methods were those described above. Photomicrography of muscle tissue sections showed diffuse myopathic lesions; the most severe lesions were fusiform as clumps beaded along the length of the muscle fiber. Interference of cell metabolism by helium probably occurred at the organelle level; the effect was a dystrophic-like myopathy in the murine skeletal muscle.

A comparison of the effect of N_2O on two different strains of rats, Sprague-Dawley and Long-Evans, was reported by Green (429); white cell counts, bone marrow and thymus analyses for RNA and DNA content were employed as bases for comparison. Normal atmosphere and one composed of 70% N_2O, 20% O_2 and 10% N_2 were used in the study. After 8 days of exposure the animals were sacrificed; 17 rats served as controls in normal

atmosphere, and 13 experimental animals in the N_2O atmosphere. The response to N_2O of white cell counts, and the RNA and DNA content of thymus and bone marrow are shown in the following figure and table:

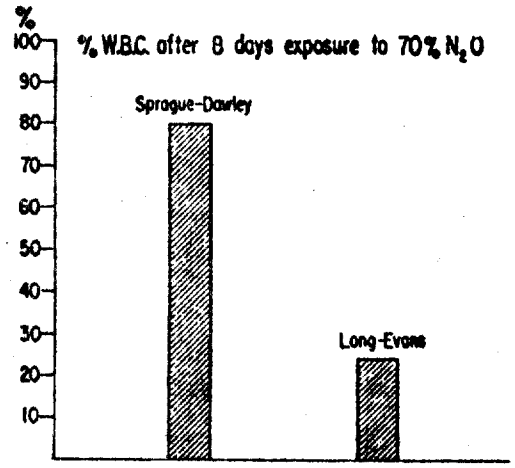

Figure 10.1. The response of the white blood cell count of adult Sprague-Dawley and Long-Evans rats is shown. After 8 days' exposure to 70% nitrous oxide, 20% oxygen and 10% nitrogen, no statistical change in the white blood cell count or differential count of Sprague-Dawley animals had occurred. Under identical testing, Long-Evans strain animals suffered a 75% decrease in white blood cell count ($p < 0.005$) and the percentage of lymphocytes increased from a normal of 72% to 95%.

TABLE 10.2

*The effect of 8 days' exposure to 70% N_2O on RNA and DNA content of organs in Sprague-Dawley and Long-Evans rats**

Strain	Content in			
	Thymus		Marrow	
	RNA	DNA	RNA	DNA
	μg/mg		μg/mg	
Sprague-Dawley				
Control	3.8 ± 0.987	22.0 ± 9.6	12.5 ± 5.83	29.4 ± 11.36
N_2O treated	4.1 ± 0.835	15.5 ± 4.23†	8.9 ± 4.45	12.7 ± 5.81†
Long-Evans				
Control	7.8 ± 2.01	22.2 ± 4.83	24.9 ± 6.72	22.8 ± 2.78
N_2O treated	14.9 ± 7.13‡	13.2 ± 7.76§	26.5 ± 11.21	10.3 ± 3.29§

* Results are expressed as mean values and standard deviation of the mean.
† $p < 0.02$.
‡ $p < 0.01$.
§ $p < 0.001$.

The difference in white cell counts in the two strains of rats is significant, with the Long-Evans animals reacting markedly (controls/experimentals). The DNA content decreased significantly in both strains, but most markedly in the Long-Evans animals. In general, it was shown that the Sprague-Dawley rats can live in 70% N_2O for 8 days without showing decreased peripheral white cell counts or in the proportion of granulocytes to lymphocytes; Long-Evans rats showed a drop to 25% of normal white cell counts and a differential predominance of lymphocytes. Small effects on RNA and DNA in Sprague-Dawley animals were seen; marked alteration of the RNA/DNA ratio occurred in the Long-Evans animals.

Recent (1972) experimental and epidemiological investigations of the effect of N_2O on the lipid metabolism of guinea pig and of man were reported by Kosmider and Misiewicz (619).

Male guinea pigs were used in the experiments: 50 animals, weighing 350-530 g, comprised the control group, breathing fresh air; 30 animals, weighing 400-590 g were exposed 8 hours daily for 180 days to an atmosphere containing N_2O in 1 ppm concentration. Body weight, total serum, free and esterified cholesterol, and total lipids were determined; liver sections were taken after sacrifice of the animals, and total liver lipids and cholesterol were determined. The results were summarized in the following table:

Table 1. The level of total, free and esterified cholesterol, total lipids and lipoprotein fractions in serum, and the content of total lipids and total cholesterol in liver homogenates of control and exposed animals

	Control group $\bar{x}_1 \pm S$ (range)	Animals exposed to nitrogen oxides $\bar{x}_2 \pm S$ (range)	$\bar{x}_1 - \bar{x}_2$	Student's t-test
Total cholesterol (mg/100 ml)	45.5 ± 8.72 30.1 — 61.0	53.42 ± 12.2 38.4 — 87.0	—7.9	2.5**
Cholesterol esters (mg/100 ml)	24.2 ± 7.15 12.0 — 39.0	28.0 ± 7.55 16.0 — 48.0	—3.8	1.7*
Free cholesterol (mg/100 ml)	21.5 ± 3.94 16.6 — 29.4	23.3 ± 10.8 8.8 — 49.0	—1.8	0.8
Total lipids (mg/100 ml)	403.0 ± 75.8 240.0 — 570.0	531.2 ± 121.5 275.0 — 730.0	—128	4.3***
α_1- + α_2-lipoproteins (%)	39.6 ± 5.19 30.6 — 50.0	40.0 ± 3.26 35.0 — 46.0	—0.4	0.33
β- + γ-lipoproteins (%)	60.4 ± 5.18 50.0 — 70.0	60.0 ± 3.25 54.0 — 64.0	0.4	0.4
Total lipids in liver homogenates (mg/100 g fresh tissue)	4475.0 ± 1369.4 2500.0 — 7000.0	4608.0 ± 1104.7 3500.0 — 6800.0	—123	0.35
Total cholesterol in liver homogenates (mg/100 g fresh tissue)	270.0 ± 42.4 200.0 — 304.0	238.0 ± 59.9 172.0 — 278.0	—32.0	2.1*

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

The serum cholesterol levels of the N_2O-treated animals showed a significant increase over control levels, as did the total lipids; liver cholesterol levels in the treated animals were significantly lower, but total lipids did not differ essentially from controls.

The epidemiological studies were performed with 70 male workers, chronically exposed to N_2O in a chemical factory. The nitrous oxide concentration in the factory ranged between 0.4 and 2.7 ppm; exposures of workers, 6 to 8 hours daily was over a period of from 4 to 6 years. Ages of workers ranged from 26 to 48 years. A control group of 80 healthy nonexposed workers in the same age group was used to compare the following parameters: total, free and esterified cholesterol, total lipids, and lipidogram. The results are shown in the following tables:

Table 2. The level of total, free and esterified cholesterol in serum of the human control group and human subjects chronically exposed to nitrogen oxides, in relation to age

Age (years)	26—36				37—48			
Population	Exposed to nitrogen oxides $\bar{x}_1 \pm S$ (range)	Control group $\bar{x}_2 \pm S$ (range)	$\bar{x}_1 - \bar{x}_2$	t	Exposed to nitrogen oxides $\bar{x}_1 \pm S$ (range)	Control group $\bar{x}_2 \pm S$	$\bar{x}_1 - \bar{x}_2$	t
Number of cases	42	42			28	38		
Total cholesterol (mg/100 ml)	180.0 ± 29.8 143.0 — 230.0	224.0 ± 28.1 160.0 — 280.0	44.0	6.9	214.0 ± 39.0 140.0 — 300.0	265.6 ± 29.6 190.0 — 316.0	51.6	5.9
Statistical significance	$p < 0.001$				$p < 0.001$			
Esterified cholesterol (mg/100 ml)	103.9 ± 24.2 64.0 — 168.0	144.8 ± 19.8 100.0 — 180.0	40.9	8.9	114.0 ± 31.6 62.0 — 197.0	165.6 ± 21.9 105.0 — 215.0	51.6	7.4
Statistical significance	$p < 0.001$				$p < 0.001$			
Free cholesterol (mg/100 ml)	76.0 ± 23.5 32.0 — 132.0	78.7 ± 19.8 35.0 — 120.0	2.7	0.6	90.9 ± 30.9 38.0 — 173.0	103.0 ± 24.8 48.0 — 195.0	3.1	0.6
Statistical significance	not significant				not significant			

Table 3. Total lipids and lipoprotein fraction in serum of the human control group and human subjects chronically exposed to nitrogen oxides in relation to age

Age (years)	26—36				37—48			
Population	Exposed to nitrogen oxides $\bar{x}_1 \pm S$ (range)	Control group $\bar{x}_2 \pm S$ (range)	$\bar{x}_1 - \bar{x}_2$	t	Exposed to nitrogen oxides $\bar{x}_1 \pm S$ (range)	Control group $\bar{x}_2 \pm S$ (range)	$\bar{x}_1 - \bar{x}_2$	t
Number of cases	42	42			28	38		
Total lipids (mg/100 ml)	718.8 ± 24.5 500.0 — 1000.0	582.7 ± 80.3 495.0 — 750.0	136.1	3.2	754.7 ± 89.2 500.0 — 900.0	598.9 ± 73.2 460.0 — 780.0	155.8	7.6
Statistical significance	$p < 0.01$				$p < 0.001$			
Lipidogram (%)								
$\alpha_1 + \alpha_2$	19.0 ± 8.0 6.0 — 39.0	31.1 ± 4.5 24.0 — 40.0	12.1	8.9	21.3 ± 7.0 13.0 — 40.0	30.9 ± 4.8 19.0 — 40.0	9.7	6.5
Statistical significance	$p < 0.001$				$p < 0.001$			
$\beta + \gamma$	80.9 ± 8.0 61.0 — 94.0	68.6 ± 4.5 60.0 — 74.0	12.3	7.1	78.8 ± 7.2 72.0 — 87.0	69.0 ± 4.8 60.0 — 81.0	9.8	6.5
Statistical significance	$p < 0.001$							

Significant differences in total and esterified cholesterol with age was observed in both control and exposed groups. Total lipid concentrations increased significantly in the exposed group compared to the controls, and the lipidogram showed significant changes - a decreased alpha 1 + alpha 2 fraction and increased beta + gamma fraction.

Chronic exposure to N_2O was demonstrated to cause disturbance in lipid metabolism in guinea pig and in man. One difference between guinea pig and man was seen in cholesterol levels, increased in guinea pig and decreased in man, by exposure to N_2O.

V. Drug Interaction

No information found

VI. Consumer Exposure Information

Consumer exposure information is restricted to the two gases, nitrogen and nitrous oxide. Both are used primarily either to exclude oxygen from packaged foods, or to serve as a propellant for sprayed or foamed food products. Some foods absorb small quantities of the gases which are then ingested.

The following tables were compiled from data submitted by user firms. Food consumption values for each food category were derived from the Market Research Corporation of America (MRCA) data on the frequency of eating, and from the USDA data on mean portion size of foods in each category. The food consumption values thus derived were coupled with the usage level data obtained in the surveys to calculate the daily intake of the substance.

Table 2 reports the usage of nitrogen and nitrous oxide in foods; table 11 reports the annual poundage data. Table 13 reports the possible daily intake and total dietary, based on food consumption by total sample.

TABLE 2 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
NITROGEN NAS 0138	04	FATS OILS(R)	*	.00145	.00406
	08	PROCSD FRUT(R)	*	.00003	.00003
	12	EGG PRODS(R)	*	.00004	.00004
	14	PROCSD VEGS(R)	*	.00000	.00000
	16	SOFT CANDY(R)	*	.01000	.01000
	20	GELATIN PUD(R)	*	.01000	.01000
	23	BEV TYPE I(R)	*	4.07000	4.07000
NITROUS OXIDE NAS 0139 FEMA 2779	23	BEV TYPE I(R)	*	.00010	.00030
	28	IMIT DAIRY(R)	*	2.27998	2.28000

TABLE 11, PART A -- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
NITROGEN NAS 0138	9/ 11	1,710,550	13,779,118	13,864,573			13,864,573
NITROUS OXIDE NAS 0139 FEMA 2779	*/ *	233,333	118,000	118,000	*	33	118,033

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBIT SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME		NO OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
NITROGEN NAS 0138	04 FATS OILS(R)	*	0-5 MO.		.007250	.007250	.020300
			6-11 MO.		.640500	.168750	.112650
			12-23 MO.		.091550	.174000	.255780
			2-65+ YR.		.255150	.450000	.710500
NITROGEN NAS 0138	08 PROCSD FRUT(R)	*	0-5 MO.		.001410	.003700	.001410
			6-11 MO.		.015540	.038700	.015540
			12-23 MO.		.030100	.059910	.030100
			2-65+ YR.		.035490	.075100	.035490
NITROGEN NAS 0138	12 EGG PRODS(R)	*	0-5 MO.	**********		.000600	**********
			6-11 MO.		.000200	.001700	.000200
			12-23 MO.		.000360	.001560	.000360
			2-65+ YR.		.000700	.002700	.000700
NITROGEN NAS 0138	14 PROCSD VEGS(R)	*	0-5 MO.		.000000	.000000	.000000
			6-11 MO.		.000000	.000000	.000000
			12-23 MO.		.000000	.000000	.000000
			2-65+ YR.		.000000	.000000	.000000
NITROGEN NAS 0138	16 SOFT CANDY(R)	*	0-5 MO.		.020000	.200000	.020000
			6-11 MO.		.220000	.680000	.220000
			12-23 MO.		.350000	.930000	.350000
			2-65+ YR.		.580000	1.760000	.580000
NITROGEN NAS 0138	20 GELATIN PUD(R)	*	0-5 MO.		.200000	.270000	.200000
			6-11 MO.		1.280000	3.880000	1.280000
			12-23 MO.		1.380000	3.360000	1.380000
			2-65+ YR.		2.040000	5.250000	2.040000

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBIT SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	FOOD CATEGORY NAME	NO OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
NITROGEN NAS 0138	20	GELATIN PUD(R)	*	6-11 MO.	1.280000	3.880000	1.280000
				12-23 MO.	1.380000	3.360000	1.380000
				2-65+ YR.	2.040000	5.250000	2.040000
NITROGEN NAS 0138	23	BEV TYPE I(R)	*	0-5 MO.	97.680000	146.520000	97.680000
				6-11 MO.	923.890000	3162.390000	923.890000
				12-23 MO.	2205.940000	6613.750000	2205.940000
				2-65+ YR.	4232.800000	11302.390000	4232.800000
NITROGEN NAS 0138	99	ALL CATEGORIES	10	0-5 MO.	97.908660	147.001430	97.921710
				6-11 MO.	925.446340	3167.098170	925.519420
				12-23 MO.	2207.791890	6618.275470	2207.956320
				2-65+ YR.	4235.710000	11309.936140	4236.166750
NITROUS OXIDE NAS 0139 FEMA 2779	23	BEV TYPE I(R)	*	0-5 MO.	.002400	.003600	.007200
				6-11 MO.	.022700	.077700	.068100
				12-23 MO.	.054200	.162500	.162600
				2-65+ YR.	.104000	.277700	.312000
NITROUS OXIDE NAS 0139 FEMA 2779	28	IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	31.919720	52.439540	31.920000
				12-23 MO.	18.239840	77.519320	18.240000
				2-65+ YR.	20.519820	34.199700	20.520000
NITROUS OXIDE NAS 0139 FEMA 2779	99	ALL CATEGORIES	*	0-5 MO.	.002400	.003600	.007200
				6-11 MO.	31.942420	52.517240	31.988100
				12-23 MO.	18.294040	77.681820	18.402600
				2-65+ YR.	20.623820	34.477400	20.832000

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Analysis of gas mixtures by gas chromatography

The purpose of this investigation was to develop a reproducible chromatographic technique for the quantitative analysis of gas mixtures containing oxygen, nitrogen, methane, nitrous oxide and carbon dioxide. The use of types 5A and 13X molecular sieves has been reported[1,2] for the separation of hydrogen, oxygen, nitrogen, methane and carbon monoxide. GRAVEN[3] developed a method, using temperature programming, for the separation of mixtures containing oxygen, nitrogen, nitrous oxide, carbon dioxide, carbon monoxide and methane. MURAKAMI[4] has analysed a mixture containing oxygen, nitrogen, methane, carbon monoxide and carbon dioxide using an arrangement of two columns in series. The gases passed first through a silica gel column to the reference cell of a thermal conductivity detector, then through a molecular sieve column and finally through the measuring cell of the detector. By suitable switching of the recorder polarity a continuous chromatogram was obtained showing a peak for the mixture of oxygen, nitrogen, methane and carbon monoxide followed by a carbon dioxide peak from the silica gel column, then individual peaks of oxygen, nitrogen, methane and carbon monoxide from the molecular sieve column. A similar technique was used by MANKA[5] for the separation of hydrogen in addition to the other gases.

Nitrous oxide and carbon dioxide have until recently proved difficult to separate by gas chromatography. Separations have been reported on columns packed with silica gel[6], charcoal[7], molecular sieve[8] and dimethyl sulphoxide on Sil-o-cel[9] but acceptable chromatograms are difficult to obtain without careful pre-treatment of the column packings. HOLLIS[10] has demonstrated that nitrous oxide and carbon dioxide are readily separated on a column packed with porous beads of a polymer made from ethylvinylbenzene with divinylbenzene as crosslinker. The work described here uses two columns in series to effect complete separation of oxygen, nitrogen, methane, carbon dioxide and nitrous oxide; the first is packed with porous polymer beads and the second with 5A molecular sieve.

Experimental

A gas chromatographic system employing two columns in series is used for the analysis so that all the components can be determined in a single sample. A length of copper tubing between the columns enables the gases which are separated on column I to be eluted before any emerge from column II. This permits the use of one detector resulting in a continuous chromatogram with no peak overlap.

The apparatus is shown diagrammatically in Fig. 1.

Column I is a 2 ft. 3 in. length of 0.25 in. O.D. copper tubing filled with 50–80

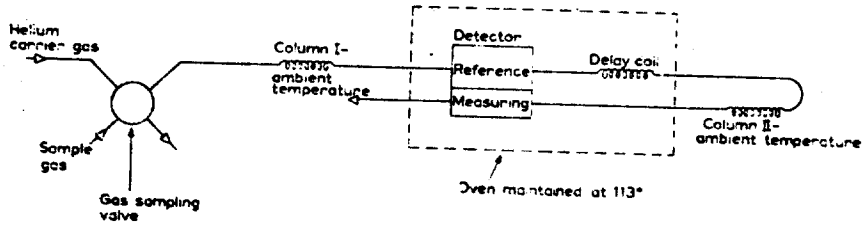

Fig. 1. Arrangement of columns.

mesh Porapak Q (Waters Associates Limited, Stockport, Ches.). This column is operated at ambient temperature.

The delay coil is a 7 ft. length of 0.125 in. O.D. copper tubing housed in the detector oven. All connections are made with compression couplings (Simplifix Limited, Maidenhead, Berks.).

Column II is a 6 ft. length of 0.25 in. O.D. copper tubing packed with 30–60 mesh 5A molecular sieve. The molecular sieve was activated prior to packing by heating at 250° for 4 h under vacuum. This column is also operated at ambient temperature.

A Gow Mac type 9285 thermal conductivity cell fitted with SS/W2 filaments was used in conjunction with a Gas Chromatography Limited RY 100 bridge unit. The detector was maintained at 113° in a Griffin and George Limited precision air thermostat oven. The bridge output was recorded on a Sunvic 0–5 mV potentiometric recorder. Helium was used as carrier gas at a flowrate of 50 ml/min.

The column arrangement uses the reference cell of the katharometer to detect gases from column I and the measuring cell to detect gases from column II. Peaks from either cell can be recorded in a continuous chromatogram by reversing the polarity of the detector signal. To achieve this without affecting the base line the recorder input signal is first adjusted to zero potential.

Samples were introduced into the chromatograph using a Perkin Elmer gas valve. Column I gives a peak representing the mixture of oxygen and nitrogen followed by individual peaks for methane, carbon dioxide and nitrous oxide. From the reference cell the gases pass on to column II which gives individual peaks for oxygen

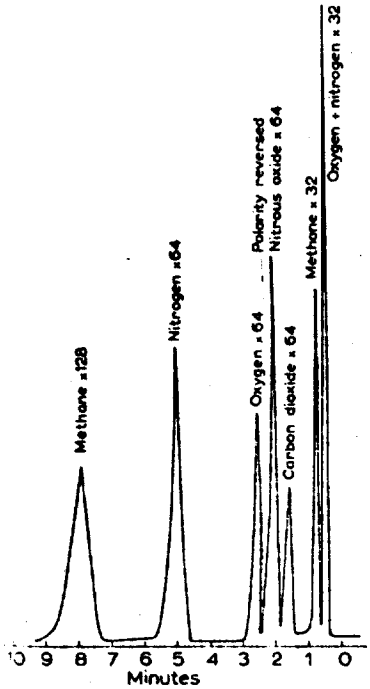

Fig. 2. Typical chromatogram for sample of gas mixture.

nitrogen and methane. Carbon dioxide and nitrous oxide are irreversibly adsorbed on this column.

Results

A typical chromatogram is shown in Fig. 2. The precision of the method was assessed by introducing twelve 1.1 ml samples of the individual gases into the chromatograph. The resulting peak areas, measured by a triangulation method, are shown in Table I.

TABLE I

PEAK AREAS FOR DUPLICATE SAMPLES OF INDIVIDUAL GASES

Helium flowrate: 50 ml/min. Sample size: 1.1 ml at N.T.P. Bridge current: 250 mA. Sensitivity corrected to × 1. Chart speed: 30 in./h. Detector temperature: 113°. Column temperature ambient.

Gas sample No.	*Methane (Porapak)*	*Methane (Molecular sieve)*	*Carbon dioxide*	*Nitrous oxide*	*Oxygen*	*Nitrogen*
1	0.291	0.277	0.304	0.418	0.288	0.328
2	0.290	0.277	0.304	0.419	0.288	0.329
3	0.291	0.277	0.305	0.419	0.288	0.324
4	0.289	0.275	0.304	0.414	0.282	0.323
5	0.290	0.277	0.306	0.418	0.282	0.321
6	0.290	0.278	0.308	0.405	0.281	0.318
7	0.291	0.275	0.304	0.417	0.281	0.324
8	0.289	0.273	0.305	0.417	0.288	0.323
9	0.289	0.277	0.304	0.414	0.288	0.321
10	0.290	0.278	0.304	0.415	0.283	0.319
11	0.289	0.276	0.306	0.423	0.283	0.321
12	0.290	0.278	0.303	0.416	0.288	0.321
Mean	0.290	0.277	0.305	0.416	0.285	0.324
σ	0.0005	0.0014	0.0015	0.004	0.0005	0.0025
Cv	0.175%	0.506%	0.498%	0.973%	0.175%	0.787%

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Department of Chemistry, Levington Research Station, Ipswich, Suffolk (Great Britain)

D. BENNETT

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SOME SYSTEMIC EFFECTS OF NITROUS OXIDE

BY

MAURICE BLOCH

Department of Neurosurgery, St. George's Hospital, London

SUMMARY

Observations made during the induction of hypothermia under general anaesthesia and during general anaesthesia at normal temperature, using nitrous oxide and oxygen together with halothane or methoxyflurane in low concentrations, indicate that nitrous oxide, used in excess of a 50 per cent mixture with oxygen, may produce appreciable depression of blood pressure and heart rate and reduction in muscle tone. It is suggested that (1) the hypotension and bradycardia sometimes observed during anaesthesia with halothane in low concentration and with nitrous oxide and oxygen may be reversed by reducing the concentration of nitrous oxide, and (2) temporary increase in muscle relaxation might be obtained, as required, by increasing the nitrous oxide to an 80 per cent mixture with oxygen.

Two observations made on patients during the induction of hypothermia under general anaesthesia have led to an investigation of the possibility of cardiovascular effects produced by nitrous oxide. The results suggest that nitrous oxide produces appreciable systemic effects, both at normal and at reduced temperatures.

METHOD

Anaesthesia. A Boyle apparatus, fitted with a Fluotec or Pentec vaporizer, was used to administer nitrous oxide and oxygen together with halothane or methoxyflurane in a semiclosed system with partial rebreathing. Flow rates for nitrous oxide and oxygen, unless otherwise stated, were 5 l./min (72 per cent) and 2 l./min (28 per cent), respectively. Premedication consisted of atropine 0.6 mg and pethidine 50 mg. Induction of anaesthesia was carried out with thiopentone, and suxamethonium chloride was used to facilitate laryngeal intubation.

Hypothermia was induced by means of a mattress-type surface-cooling unit.

Blood pressure was recorded with an ordinary clinical sphygmomanometer, *heart rate* with a stethoscope at the apex, and *temperature* by a thermistor placed 3–4 inches into the rectum.

The patients, who were suffering from cerebral astrocytoma, were cooled prior to irradiation to the brain. Each had recently (1–2 weeks) had a major cerebral decompression and was from a specially selected group of patients, aged 20 to 50 years, conscious, rational, and ambulant, and having only minimal abnormalities to be found on clinical examination of the central nervous system.

During induction of hypothermia under general anaesthesia, using nitrous oxide and oxygen together with halothane, methoxyflurane or ether, blood pressure, heart rate and respiratory rate fall. When active cooling and anaesthesia are discontinued the body temperature continues to fall owing to the phenomenon of "after-drop". It was noticed, however, that when anaesthesia alone is terminated (not active cooling), blood pressure and heart rate rise rapidly, often within 5 minutes. The fall in blood pressure and heart rate, therefore, are not related solely to cooling.

A second observation appeared to link this rapid rise in blood pressure and heart rate to the action of nitrous oxide.

A woman, aged 25 years, was anaesthetized with methoxyflurane 1 per cent and nitrous oxide and oxygen at flow rates of 5 l./min and 2 l./min respectively (fig. 1). Cooling was commenced after 5 minutes and the methoxyflurane was reduced to 0.5 per cent after 12 minutes. Systolic blood pressure fell to 78 mm Hg after 80 minutes and remained at this level despite reduction of the methoxyflurane concentration to 0.3 per cent. During the last 35 minutes of anaesthesia nitrous oxide alone was used but the

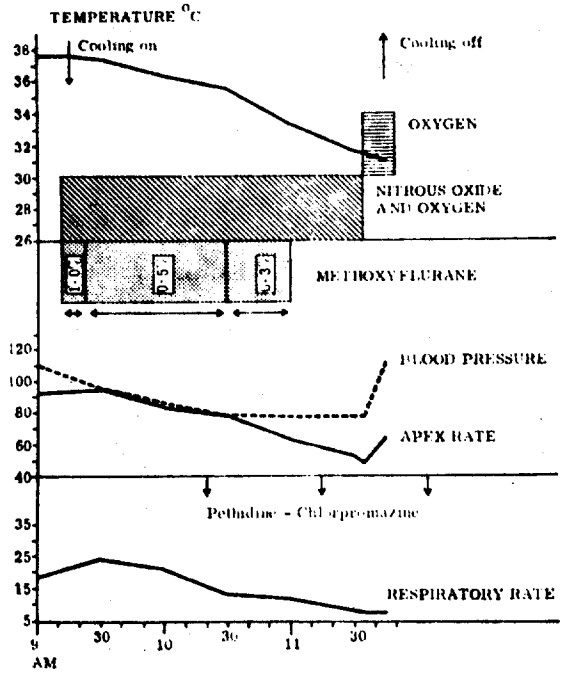

FIG. 1
E.C., Female, aged 25 years (12.11.62).

systolic blood pressure remained around 78 mm Hg and the heart rate continued to fall, largely in parallel with the fall in temperature. When nitrous oxide was discontinued (cooling unchanged) both heart rate and blood pressure rose within minutes, muscle tone increased and shivering commenced, in contrast to the preceding 35 minutes during which there had been no decrease in the depth of anaesthesia or rise in heart rate or blood pressure.

RESULTS

During further observations nitrous oxide and oxygen were replaced for short periods during anaesthesia by oxygen alone (fig. 2) or by air (fig. 3), at 7 l./min, both during cooling and before active cooling was commenced (figs. 4 and 5). The concentration of methoxyflurane (or halothane) was maintained unchanged at 0.5 per cent of the inhaled vapour. On each occasion, independently of temperature and within a few minutes of nitrous oxide withdrawal, the blood pressure rose, muscle tone increased, goose-flesh appearance became widespread and there was gagging on the endotracheal tube. The heart rate also increased, though not as constantly as did the blood pressure. These changes, which occurred also when anaesthesia was terminated (figs. 1 to 4), could be reversed within a few minutes by resumption of nitrous oxide breathing. The temporary rise in heart rate and blood pressure recorded at figures 3a, 4b and 5b is due to widespread superficial vasoconstriction resulting from placing the patient on a cooling mattress at surface temperature 7–10°C. This was missed previously (figs. 1 and 2) owing to a longer interval between observations made during the early part of anaesthesia.

Similar observations were made, in all, on thirty occasions during fifteen anaesthetics administered to six patients. The volatile anaesthetics remaining after nitrous oxide withdrawal were halothane (eight), methoxyflurane (six) and ether (one). Of eight observations made before cooling, five concerned halothane, two methoxyflurane and one ether, and of twenty-two made during cooling, at temperatures which varied from 29.7 to 36.1°C, twelve concerned halothane, nine methoxyflurane and one ether. The intervals during which nitrous oxide was withdrawn varied in duration from 5 to 45 minutes.

The systolic blood pressure rose during twenty-eight (93 per cent) of the thirty observations (table I). There was no appreciable difference between the cooled and the uncooled patients or between the effects of the volatile anaesthetics that remained. The elevation of systolic blood pressure was of the order of 7–10 mm Hg on seven occasions, 11–20 mm Hg (eleven), 21–30 mm Hg (six), 31–40 mm Hg (two) and 41–50 mm Hg (two). On the remaining two occasions the rise was too small to be significant. The maximum rise occurred within 5 to 10 minutes of withdrawal of nitrous oxide and was independent of the interval during which the nitrous oxide was withdrawn, and, after approximately the first hour, of the duration of anaesthesia.

The heart rate increased on eighteen (62 per cent) of twenty-nine instances on which it was observed (table I). The rise was of the order of less than 10 beats/min on three occasions, 11–20 beats/min (seven), 21–30 beats/min (four) and over 40 beats/min (four), and was independent of temperature, the particular volatile gas remaining after nitrous oxide withdrawal and after approximately 1 hour, the duration of anaesthesia.

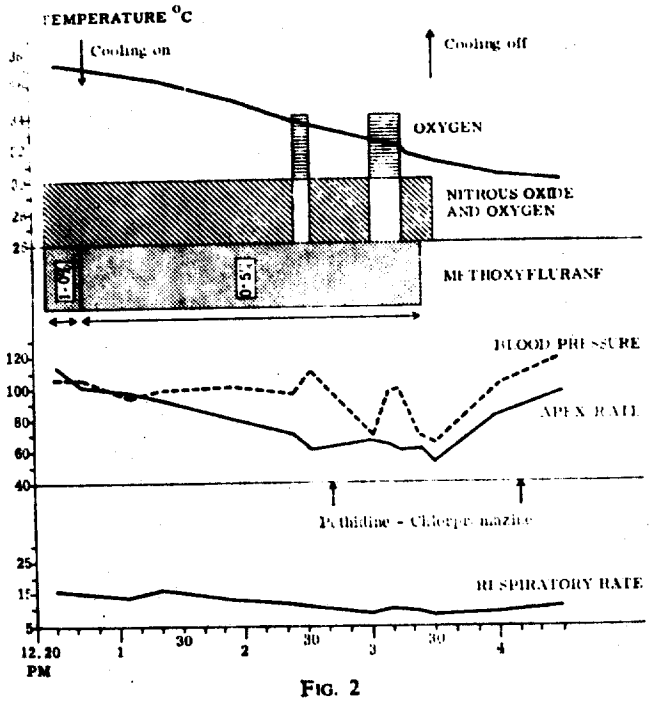

FIG. 2
T.H., female, aged 35 years (19.11.62).

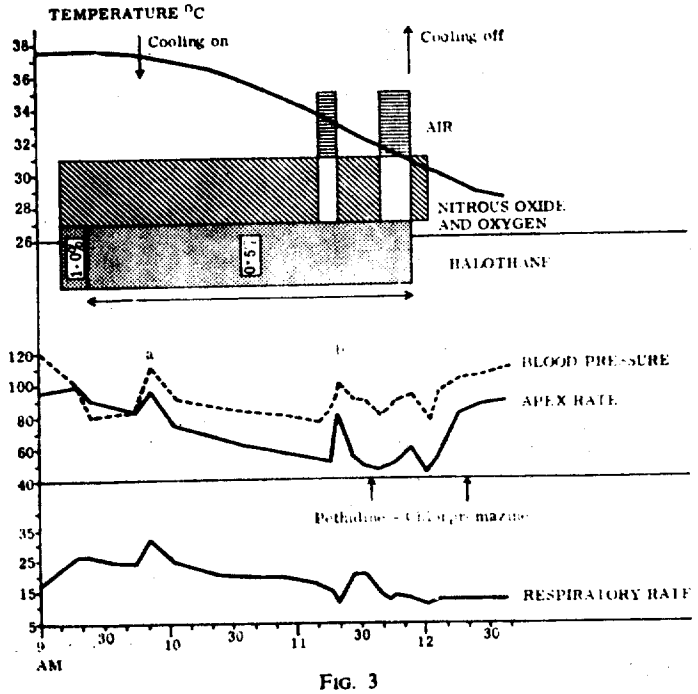

FIG. 3
E.C., female, aged 25 years (10.12.62).

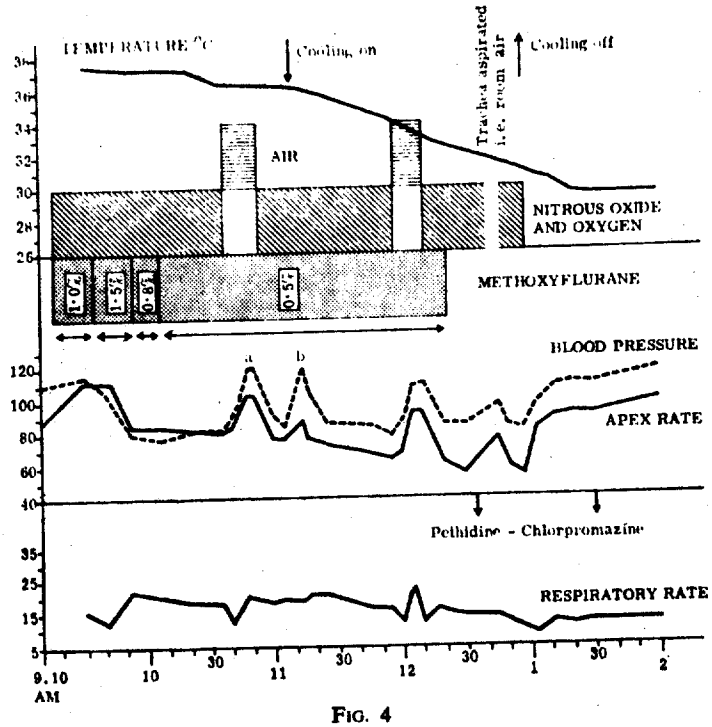

FIG. 4
T.H., female, aged 35 years (2.1.63).

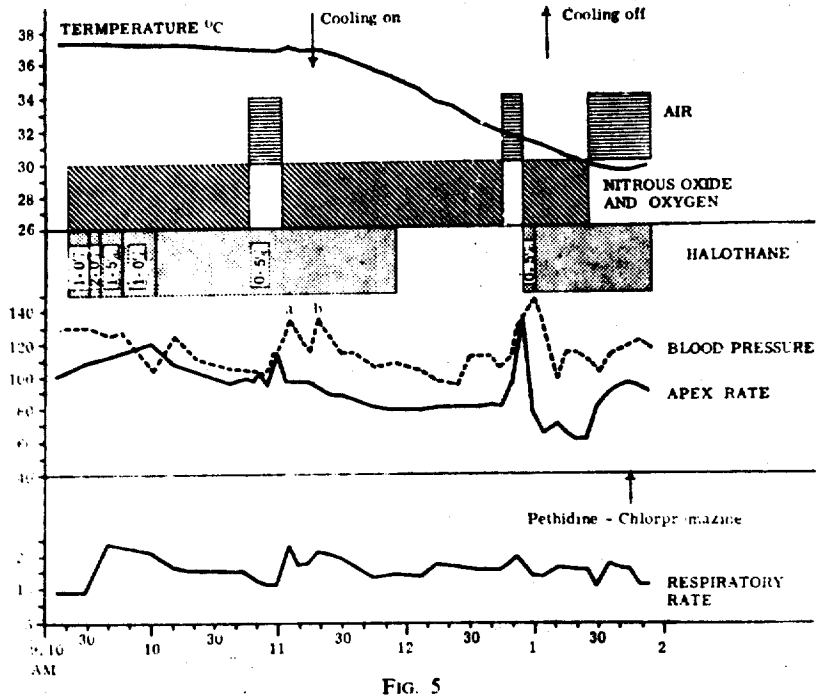

FIG. 5
T T., male, aged 37 years (16.1.63).

TABLE I

Effect on blood pressure, heart rate and respiratory rate of withdrawing nitrous oxide and leaving halothane (H), methoxyflurane (M), or ether (E)

Blood pressure	Number of observations	Rise in blood pressure	Rise less than 7 mm Hg	7-10 mm Hg	11-20 mm Hg	21-30 mm Hg	31-40 mm Hg	41-50 mm Hg
Uncooled	8	7	1 H-1 M-0 E-0	3 H-2 M-0 E-1	2 H-1 M-1 E-0	1 H-1 M-0 E-0	1 H-0 M-1 E-0	0 H-0 M-0 E-0
Cooled	22	21	1 H-1 M-0 E-0	4 H-2 M-2 E-0	9 H-6 M-3 E-0	5 H-2 M-2 E-1	1 H-0 M-1 E-0	2 H-1 M-1 E-0
Total	30	28	2	7	11	6	2	2

Heart rate	Number of observations	Rise in heart rate	Less than 10 beats/min	11-20 beats/min	21-30 beats/min	31-40 beats/min	Greater than 40 beats/min	Fall in heart rate	Rate unchanged
Uncooled	8	6	1 H-1 M-0 E-0	2 H-1 M-0 E-1	1 H-0 M-1 E-0	0 H-0 M-0 E-0	2 H-1 M-1 E-0	2 H-2 M-0 E-0	0 H-0 M-0 E-0
Cooled	21	12	2 H-0 M-2 E-0	5 H-3 M-2 E-0	3 H-2 M-1 E-0	0 H-0 M-0 E-0	2 H-2 M-0 E-0	8 H-5 M-3 E-0	1 H-0 M-0 E-1
Total	29	18	3	7	4	0	4	10	1

Respiratory rate	Number of observations	Fall in respiratory rate	Fall less than 4 b.p.m.	4-6 b.p.m.	7-9 b.p.m.	Rise in respiratory rate	Rate unchanged
Uncooled	8	4	3 H-2 M-1 E-0	3 H-2 M-1 E-0	1 H-0 M-0 E-1	1 H-1 M-0 E-0	0 H-0 M-0 E-0
Cooled	17	10	3 H-2 M-1 E-0	6 H-4 M-2 E-0	4 H-3 M-0 E-1	1 H-0 M-1 E-0	3 H-1 M-2 E-0
Total	25	14	6	9	5	2	3

On nine occasions during cooling (five halothane, three methoxyflurane, one ether) and twice before cooling (halothane) the heart rate either fell (fig. 2) or remained unchanged following nitrous oxide withdrawal. The changes were all of the order of 12 beats/min or less.

Changes in respiratory rate on withdrawing nitrous oxide were more difficult to assess but excluding those in which there was a rise (two), or in which the change in rate was less than 4 b.p.m. or was not sustained throughout the period of nitrous oxide withdrawal, the respiratory rate fell on fourteen (56 per cent) out of the twenty-five instances recorded (table I). The most impressive changes in rate were of the order of 6-9 b.p.m., e.g. from 14 down to 7 b.p.m. (halothane, temperature 33.4 C), from 18 down to 12 b.p.m. (methoxyflurane, uncooled), from 25 down to 16 b.p.m. (ether, 32.5°C).

The effect of gradually diminishing the concentration of nitrous oxide in the inspired vapour (the halothane remaining unchanged) was assessed

by reducing the proportion of nitrous oxide to oxygen, in turn, from 5:2 to 4:3 to 3:4 and to 2:5 the total flow rate remaining unchanged. After each such change a return to the original proportions of these gases was made for a period of 15 to 20 minutes before proceeding to a further change. The effect of changing the proportions of the gases from 5:2 to 4:3 was minimal and unconvincing. At 3:4 the effect was more impressive. Systolic blood pressure rose from 78 to 96 mm Hg, heart rate fell from 54 to 48 beats/min and respiratory rate from 23 to 20 b.p.m. but there was no increase in muscle tone or evident lightening of anaesthesia. The most marked changes occurred when the proportions of gases were reversed from 5:2 to 2:5. Systolic blood pressure rose from 78 to 94 mm Hg, heart rate from 48 to 68 beats/min and respiratory rate fell from 22 to 18 b.p.m. There was marked increase in muscle tone and there were movements in all four limbs. These changes were rapidly reversed by restoring the original proportions of the gases.

Withdrawal of the volatile gas, leaving only nitrous oxide and oxygen in the breathing circuit (figs. 1, 4 and 5) was carried out eight times (halothane, four; methoxyflurane, four) in four patients at temperatures which varied from 30.6 to 36.2°C and for periods of from 22 to 52 minutes.

The heart rate remained unchanged or continued to fall on all eight occasions and the blood pressure on seven. The respiratory rate showed little change on three occasions but on the other five a marked fall was recorded, e.g. from 15 to 8 b.p.m., 13 to 5 b.p.m., 11 to 7 b.p.m. Slight increase in muscle tone and in depth of respiration was noted on two occasions.

On two of these eight occasions further observations were made. After the volatile gas had been withdrawn without producing significant changes in blood pressure, heart rate or respiratory rate, the nitrous oxide also was withdrawn, leaving the patient breathing only air at 7 l./min. On the first occasion (temperature 29.7 C) nitrous oxide was withdrawn 27 minutes after methoxyflurane. Within 3 minutes muscle tone had increased and after 5 minutes systolic blood pressure had risen from 120 mm Hg to 135 mm Hg., and heart rate from 72 beats/min (regular) to 132 beats/min (fibrillating). Nitrous oxide breathing was resumed after a further minute and was followed by rapid reversal of these changes. On the second occasion (fig. 5, same patient but using halothane) nitrous oxide was withdrawn 52 minutes after halothane and resulted in a similar pattern of changes. On each occasion the withdrawal of nitrous oxide resulted in a grossly irregular, rapid heart beat which reverted to normal within 5–10 minutes of resuming nitrous oxide breathing.

On the first occasion, after a further interval of 18 minutes breathing nitrous oxide and oxygen the nitrous oxide was withdrawn with the intention of terminating anaesthesia. Blood pressure and heart rate rose and the heart rhythm again became grossly irregular at 128–140 beats/min. In view of the persistence of this irregularity over a period of 50 minutes, nitrous oxide was re-introduced into the breathing circuit. The cardiac rhythm did not revert to normal but over a period of 15 minutes the rate fell steadily from 140 to 132, 128, 116 beats/min. Unfortunately, by this time the temperature was noted to be rising and the situation was complicated by the necessity for further cooling.

On the remaining six occasions the volatile gas was withdrawn towards the end of anaesthesia (figs. 1, 3 and 4) and was followed after intervals of 8, 21, 26, 31, 35 and 36 minutes, respectively, by termination of anaesthesia and withdrawal of the nitrous oxide, the patients breathing oxygen, room air or air at 7 l./min obtained from an air cylinder. In all six the systolic blood pressure and the heart rate, which had not risen during the withdrawal of the volatile gas, rose within a few minutes by 10–40 mm Hg and 16–54 beats/min, respectively. It is interesting that in six of these nine episodes the respiratory rate either remained unchanged (fig. 1) or increased (fig. 5) but did not fall. In three the respiratory rate was not observed.

Thus, of a total of thirty-nine observations on blood pressure a definite rise was recorded in thirty-seven (95 per cent). Heart rate rose during twenty-seven (71 per cent) of thirty-eight observations. The respiratory rate fell by 4–9 b.p.m. on fourteen (45 per cent) and rose by 4–9 b.p.m on five (16 per cent) of thirty-one observations.

DISCUSSION

Evidence has been presented, both at normal temperature and during the induction of hypothermia, that the depression of blood pressure and heart

rate noted during prolonged anaesthesia with nitrous oxide and oxygen together with ether or with halothane or methoxyflurane in low concentrations (0.5 per cent of the inspired vapour) may be relieved within a few minutes by removing only the nitrous oxide. Of twenty-eight such observations in which the systolic blood pressure rose on withdrawing nitrous oxide, twenty-one exceeded 10 mm Hg and ten exceeded 20 mm Hg. The heart rate rose on eighteen of twenty-nine observations—on fifteen by more than 10 beats/min and on eight by more than 20 beats/min. These changes may be reversed by resuming nitrous oxide breathing. The effect on blood pressure and heart rate of withdrawing halothane (or methoxyflurane) in the concentrations stated, and leaving only nitrous oxide and oxygen in the breathing circuit, for 20–50 minutes, is negligible when compared with that produced by withdrawing nitrous oxide for only a few minutes and leaving the volatile gas and oxygen (or air) in the breathing circuit. Further, the nitrous oxide effect may still be demonstrated 50 minutes after the withdrawal of halothane (fig. 5).

These observations suggest that changes in heart rate and blood pressure may occur as a result of anaesthesia with nitrous oxide alone, irrespective of other anaesthetic agents used. Initially observations were made during the induction of hypothermia and it was considered that they could be accounted for by the action of nitrous oxide in suppressing shivering. However, in view of the fact that similar effects have been observed on eight occasions prior to cooling, a more direct effect of nitrous oxide on cardiac function appears to be at least a possibility. In the dog heart-lung preparation (Price and Helrich, 1955) this has been demonstrated to be the case but clearly further observations, on the intact animal and on man, are necessary.

Lassen and colleagues (1956), who described fatal bone marrow depression in association with the prolonged use of nitrous oxide in the treatment of tetanus, made no reference to hypotension, despite the continuous use of the gas for periods of sixteen and eighteen days. However, these workers used a 50 per cent mixture of nitrous oxide with oxygen and the absence of hypotension is in keeping with the present observation that depression of blood pressure was obtained when the proportion of nitrous oxide to oxygen was 5:2 or 4:3 (57:43 per cent) but not 3:4 or 2:5.

It may be argued that depression of blood pressure results from the extensive vasodilatation known to accompany general anaesthesia and that any reduction in the depth of anaesthesia would result in a rise in blood pressure. However, observations were carried out at a temperature as low as 29.7 C and under these conditions intensive widespread superficial vasoconstriction is present, both in the anaesthetized and in the conscious patient.

The rise in blood pressure is not necessarily related to increase in muscle tone, since frequently it preceded clinically detectable change in muscle tone and even occurred in its absence (i.e. when breathing a 3:4 mixture of nitrous oxide with oxygen). It is interesting that Bodman, Morton and Thomas (1955), reporting the treatment of tetanus with chlorpromazine and nitrous oxide, noted that muscle tone and convulsions returned when nitrous oxide was withdrawn. However, the flow rates of nitrous oxide and oxygen are not stated in their paper.

One result of these observations may be that the correction of the hypotension and bradycardia sometimes seen during anaesthesia with nitrous oxide and a low concentration of halothane may lie as much with withdrawal of the nitrous oxide as with the use of atropine or withdrawal of halothane.

Respiratory changes were less constant but it is of interest that the withdrawal both of nitrous oxide and of halothane (or methoxyflurane or ether) is capable of causing a significant fall in respiratory rate. Eckenhoff and Helrich (1958) reported that nitrous oxide acting alone appeared to "stimulate respiration slightly". Deutsch and colleagues (1962) described the effects of halothane acting alone and reported that "during the first 31–54 minutes of anaesthesia cardiac output and arterial pressure decreased but with the passage of additional time (up to 129 minutes) cardiac output returned to pre-anaesthetic values and heart rate increased above control values. Respiratory rate . . . was increased during the entire period of halothane anaesthesia". These

findings may account for the following observations reported in the present paper:

(1) Only small changes in blood pressure and heart rate occurred on withdrawal of nitrous oxide during the first hour of anaesthesia. Possibly the effects of nitrous oxide withdrawal were obscured by the depressant effects of halothane persisting during this period.

(2) Withdrawal of halothane, in the presence of nitrous oxide, was not followed by elevation of blood pressure and heart rate. The earliest occasion on which this was carried out was after 110 minutes of anaesthesia, but by this time, according to Deutsch et al., the depressant effects of halothane, even in higher than maintenance concentration, would have been reversed. This lends further support to the contention that the changes in blood pressure and heart rate present at that time were related to nitrous oxide.

(3) The fall in respiratory rate following halothane withdrawal.

Further evidence implicating nitrous oxide in the changes described in this paper comes from Severinghaus (1954) and Frumin, Salanitre and Rackow (1961) who investigated the elimination of nitrous oxide and reported, respectively, that arterial blood loses 70 per cent of its nitrous oxide in 3 minutes and 90 per cent in 20 minutes and that the end-tidal expiratory nitrous oxide concentration fell from 74 to 22 per cent in 2 minutes and to 9.5 per cent in 10 minutes. These intervals, which are similar to those taken for nitrous oxide uptake, are in keeping with the rapid onset of changes reported, in this paper, to follow on the withdrawal of and re-introduction of nitrous oxide during light anaesthesia.

Finally, brief reference is made to the changes in cardiac rhythm noted on three occasions, in the same patient, following cessation and resumption of nitrous oxide breathing. On withdrawing the nitrous oxide the heart rate changed within a few minutes from a regular rhythm at 80 (fig. 5), 72 and 72 beats/min to a grossly irregular rhythm at 134, 132 and 128 beats/min, respectively. No e.c.g. check was made but the irregularity was considered on clinical grounds to be due to auricular fibrillation. On two occasions the cardiac rate and rhythm reverted to normal within 5-10 minutes of resuming nitrous oxide breathing, but on the third ocasion only slowing of the rate was noted. It is tempting to relate these changes to temperature (31.6, 29.7 and 29.2°C) but this type of irregularity does not normally occur above 28-30°C. In figure 5 it is seen that both nitrous oxide and halothane were returned to the respiratory circuit at the same time but on the other two occasions only nitrous oxide and oxygen were used. It would be interesting to observe the effect on patients with auricular fibrillation or paroxysmal tachycardia of breathing nitrous oxide.

CONCLUSIONS

Observations, at normal temperature and during cooling, are reported on patients undergoing semi closed circuit anaesthesia using nitrous oxide and oxygen together with halothane (0.5 per cent), methoxyflurane (0.5 per cent) or ether.

Depression of blood pressure and heart rate noted under these conditions may be relieved within a few minutes by withdrawing only the nitrous oxide. This is accompanied by reduction in the depth of anaesthesia. These changes may be reversed by resuming nitrous oxide breathing. They are not produced, even after 50 minutes, by withdrawing only the halothane or methoxyflurane.

The time taken for these changes to become detectable, following cessation or resumption of nitrous oxide breathing, is similar to that calculated for the elimination and uptake respectively of nitrous oxide by arterial blood.

Withdrawal of nitrous oxide or halothane or methoxyflurane resulted in slowing and increased depth of respiration in over 50 per cent of observations made.

It is postulated that: depression of blood pressure, slowing of the heart rate and decrease in muscle tone may be produced by nitrous oxide when used in excess of a 50 per cent mixture with oxygen; the hypotension and bradycardia sometimes observed during anaesthesia with halothane in low concentrations and with nitrous oxide and oxygen may be reversed by reducing the concentration of nitrous oxide; and temporary increase in muscle relaxation might be obtained, as required.

by increasing the nitrous oxide to an 80 per cent mixture with oxygen.

Brief reference is made to changes in cardiac rhythm noted in relation to nitrous oxide breathing.

Further observations should be made of the effects on heart rate and blood pressure of prolonged anaesthesia using nitrous oxide gas alone.

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The observations reported in this paper were made at the Royal Marsden Hospital while working as a member of the Joint Atkinson Morley (St. George's Hospital) and Royal Marsden Hospitals team investigating the problem of irradiation under hypothermia as a treatment for cerebral astrocytomata.

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CERTAINS EFFETS INTERNES DU PROTOXYDE D'AZOTE

SOMMAIRE

Des observations faites pendant anesthésie générale sous hypothermie ou sous température générale, avec emploi de protoxyde d'azote et oxygène ensemble avec halothane et méthoxyflurane, à concentrations faibles, montrent que le protoxyde d'azote utilisé dans un mélange dépassant 50% avec de l'oxygène, est susceptible de produire une dépression notable de la tension artérielle et du rendement du coeur ainsi qu'une réduction du tonus musculaire. L'auteur suggère (1) que l'hypotension et la bradycardie observes quelquefois pendant des anesthésies à l'halothane à concentration faible et avec du protoxyde d'azote et oxygène, peuvent être inverssées par réduction de la concentration de protoxyde d'azote, (2) que l'on peut obtenir l'augmentation musculaire temporaire du relâchement, en augmentant selon ses besoins le taux de protoxyde d'azote jusqu'à 80% du mélange avec de l'oxygène.

EINIGE ALLGEMEINWIRKUNGEN DES LACHGASES

ZUSAMMENFASSUNG

Beobachtungen während der Einleitung der Hypothermie unter Allgemeinnarkose und während Allgemeinnarkosen bei normaler Körpertemperatur, wobei Lachgas und Sauerstoff zusammen mit Halothane oder Methoxyfluran in niedrigen Konzentrationen angewendet wurden, sprechen dafür, daß Lachgas in einer höheren als 50-prozentigen Mischung mit Sauerstoff einen merklichen Abfall des Blutdruckes und der Herzrate und ein Nachlassen des Muskeltonus hervorrufen kann. Es wird behauptet, daß erstens die manchmal bei Narkosen mit Halothane in niedrigen Konzentrationen zusammen mit Lachgas und Sauerstoff beobachtete Hyptonie und Bradykardie durch Verminderung der Lachgaskonzentration beseitigt und daß zweitens eine vorübergehende Erhöhung der Muskelerschlaffung, falls notwendig, durch Steigerung der Lachgaskonzentration bis zu einer 80-prozentigen Mischung mit Sauerstoff erreicht werden kann.

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Blodgett-'59
BIB NO. 109

Nitrous Oxide and "Freon C318"

as propellants for aerosol

AEROSOL packaging of nonfood products has grown very rapidly since its beginnings around 1947. Over the same period, growth of aerosol food products has been steady, but much slower.

The aerosol food market is potentially very large, and the next few years should show a faster growth rate than we have seen in the past decade. A number of problems have depressed the growth rate of aerosol food products, but some of these are now being resolved.

The choice of a proper propellant is critically important for a successful aerosol product. In the past, only two propellants—nitrous oxide and carbon dioxide—have been available for edible sprays or foams, and only one propellant—nitrogen—for edible nonaerated liquids.

"Freon-C318"* propellant, a new fluorocarbon, has recently become available for experimental trial in aerosol food products. Both by itself, and in combination with nitrous oxide, "Freon-C318" offers important advantages in aerosol foods.

Whipped cream, the major aerosol food product, has certain deficiencies which have retarded its market growth. "Freon-C318" may be used to make a whipped cream with markedly improved stiffness and stability, with better color retention and much less weeping or drainage, as compared to similar cream whipped with nitrous oxide. Mixtures of nitrous oxide containing 15-30 weight percent of "Freon-C318" propellant also show these quality improvements with whipped cream. Furthermore, the mixed propellant is more economical than straight "Freon-C318," and provides the higher pressures needed to dispense refrigerated or viscous food products. One important advantage—uniform consistency during use of the dispenser—has so far been attained only by using straight "Freon-C318."

The conventional "Freon" propellants used for nonfood aerosol products are not suitable for food aerosols because they usually impart an objectionable taste. All of the "Freon" propellants decompose in contact with flame or very hot surfaces, forming irritating and toxic breakdown products.

The Food Additives Amendment of 1958 requires the manufacturer of a new food additive to test it for safety on animals. After review of the test data, the Food and Drug Administration will issue regulations covering commercial use of the additive in foods.

Nitrous oxide, carbon dioxide and nitrogen have already been approved for use in aerosol food products. The required tests on "Freon-C318" have been completed. They show no effects on animals after 90 six-hour exposures to concentrations of 10% "Freon-C318" by volume in air. This is a much higher level of exposure than feeding tests could provide because of the low solubility of "Freon-C318" in water and in oil.

The animal toxicity and chemical stability data on "Freon-C318" have been reviewed with officials of the Food and Drug Administration. Their formal action may be expected within the next few months.

In addition to federal regulations, state and local boards of health may impose their own regulations to be sure that the finished aerosol food product will be clean and wholesome. This will probably mean completely separate loading lines used only for food. The food will usually have to be sterilized or pasteurized by cooking or by flash heat exchangers, and then be chilled and loaded aseptically into sterile cans. The propellant is probably already sterile, but, if necessary, it can be sterilized by filtration before loading. It will not be practical to sterilize the finished pressure package.

Nitrous oxide and carbon dioxide can be used only as compressed gas propellants. Their vapor pressures at ordinary temperatures are far too high to permit them to liquefy in an aerosol container. They do, however, dissolve to some extent in both the aqueous and the oil phases of cream, mayonnaise or other emulsified food product.

The amount of compressed gas dissolved in the food product depends on the pressure and the temperature at equilibrium. This means that the amount of propellant gas available for whipping the product when it is dispensed depends critically on the original product fill and headspace, the charging temperature and pressure, and the increasing headspace or decreasing pressure as the product is used. Foamed food products propelled with compressed gases typically show decreasing product stiffness and over run in successive portions from the same container. With some products this lack of uniformity is quite objectionable.

* Freon-C318 is du Pont's registered trademark for octafluorocyclobutane.

food products*

By F. W. Blodgett
E. I. du Pont de Nemours & Co., Inc.
Wilmington, Del.

R. C. Webster
Air Reduction Co., Inc.
Madison, Wisc.

"Freon-C318" is a liquefied compressed gas with a vapor pressure of 25 psig at 70°F. When it is used as the propellant for emulsified food products, droplets of liquefied propellant are emulsified in the aqueous phase, in addition to the propellant dissolved in the oil phase. "Freon-C318" is essentially insoluble in water. The pressure in the container, and the amount of propellant dissolved in the oil phase remain constant as long as some droplets of liquid propellant remain. Since the volume of the propellant increases by a factor of 175 as the liquid propellant evaporates, most of the "Freon-C318" remains emulsified in the product in spite of increasing headspace. For these reasons, the consistency and overrun of the whipped product remains very nearly constant during the use of a dispenser pressurized with "Freon-C318" liquefied propellant.

In 1958, a development project conducted jointly by Air Reduction and du Pont indicated that even minor concentrations of "Freon-C318" in nitrous oxide propellant give improved quality in whipped cream and other aerosol food products as compared to similar products using straight nitrous oxide propellants. Further work has shown that mixtures of "Freon-C318" and nitrous oxide form a homogeneous liquid solution in high-pressure cylinders. This makes it practical to use the mixed propellant commercially. The solution, of specified composition, is taken from the cylinder liquid phase, vaporized, passed through a reducing valve, and charged to the cans on a standard gasser-shaker. Under these conditions, the mixture is used as a compressed gas propellant at about 100 psig.

The properties of nitrous oxide and of "Freon-C318" are shown in Table I above. In spite of their dissimilarities, the two compounds are miscible in all proportions at temperatures down to about —110°F. under their own vapor pressure.

The advantages of using the mixture of nitrous oxide and "Freon-C318" rather than straight nitrous oxide are shown by the following data on whipped cream.

Two cream formulations, differing mainly in butterfat content, but adjusted to similar taste, were used:

Component	*Formula A*	*Formula B*
Butter fat	30.00%	20.00%
Serum solids	7.50	8.50
Cane sugar	7.50	6.00
Corn syrup solids	—	2.50
Protein stabilizer	0.25	0.30
Total solids	42.25	37.30

The 211 x 413 aerosol containers, of nominal 12-ounce capacity, were filled with 7 fluid ounces of a cream formulation. Valves were applied with a vacuum crimper. Straight nitrous oxide or a mixture of nitrous oxide and "Freon-C318" was then charged, with agitation, to an equilibrium pressure of 95-100 psig at 40°F. Triplicate samples were used.

Consistency, or stiffness, was measured with a curd tension meter, which determines the force in grams on a disk 1.5 inches in diameter which is supported on the whipped cream. Successive readings were taken as the disk slowly penetrated the whipped cream over a period of five minutes.

Drainage, or serum loss, was measured by placing a weighed portion of the whipped cream in a funnel over a 0.5-inch disk of 16-mesh wire screen. The sample was stored for

Table I

PROPERTIES OF PROPELLANTS

Property	*Units*	*Nitrous Oxide*	*"Freon-C318"*
Formula		N_2O	$CF_2CF_2CF_2CF_2$
Molecular Weight		44.016	200.04
Color		None	None
Odor		None	None
Taste		Slight Sweet	None
Boiling point, 1 Atm.	°F	—127.24	21.1
Density, 70°F			
Liquid	lbs./cu. ft.	46.5	94.5
Saturated vapor	lbs./cu. ft.	0.2809	1.505
Vapor pressure, 70°F	psig	745.3	25.4
Heat of vaporization, 70°F	BTU/lb.	77.7	46
Surface tension, 70°F	dynes/cm	—	8.3
Solubility parameter, 77°F		4.2	5.6

* Presented at the Mid-year Meeting, Chemical Specialties Manufacturers Association, in Chicago on May 19, 1959.

one hour at 70°F. and the drainage was recorded in ml/100 g.

Overrun and the general appearance of the whipped cream after storage at 70° F. was also recorded.

The stiffness and stability results are shown in Table II. The most marked difference is shown by the samples containing "Freon-C318." All of them show firmer consistency and better stability than those containing straight nitrous oxide. There is relatively little effect of different butterfat contents in these formulations. This shows that a whipped cream with about 30% increase in firmness can be made by using a propellant containing 15% by weight of "Freon-C318" in nitrous oxide, even though the butterfat content of the cream is reduced from 30% to 20%.

This firmer consistency and better stability probably results from the finer, more uniform bubble structure developed in whipped cream by propellants containing "Freon-C318." The very low solubility of "Freon-C318" in water is important in maintaining the uniformity of the bubble size. Soluble gas tends to pass from smaller bubbles to larger bubbles and out to the air because of the differences in pressure. "Freon-C318" is practically insoluble in water, with a solubility of about 0.006 ml/ml at 77°F. and 1 atm. This is about 1% of the solubility of nitrous oxide in water.

Overrun and drainage data are shown in Table III above. The effect of "Freon-C318" added to nitrous oxide is to increase the overrun and decrease the drainage. This is consistent with the increased foam stability shown in Table II.

Again it was noted that the whipped cream samples showing high overruns with nitrous oxide/"Freon-C318" propellant had relatively uniform, small bubbles, rather than the large gas cells often seen with straight nitrous oxide whipped creams at high overruns.

Visual observation of the whipped cream samples for softening of the edges, weeping, discoloration and collapse at 70°F. shows that samples containing nitrous oxide/"Freon-C318" propellant are definitely superior to those containing straight nitrous oxide. This is true at both butterfat levels. The effect is striking at 30 minutes and at an hour. Even after 24 hours, the samples containing "Freon-C318" retain about half their original shape and general appearance. After several hours, the samples containing straight nitrous oxide have collapsed and seriously discolored.

These data show that whipped cream formulations made with nitrous oxide and "Freon-C318" propellant are much more satisfactory than those made with straight nitrous oxide. They have better consistency, better stability, better retention of creamy-white color and less weeping or drainage.

For the first time, using this new propellant mixture, aerosol whipped cream can be used for topping desserts or other food an hour or so before it is to be served. Previously, this was a last-minute chore. In these days when convenience and labor costs are so important, these improved toppings will be welcomed by the housewife and by the chef. The new whipped creams are so stable that they can be used to top both desserts which are to be refrigerated before serving and desserts which are served hot. In the latter case, the dessert should be served promptly after topping.

There are a number of food products besides whipped cream which are suitable for packaging in aerosol form. Mayonnaise and other salad dressings operate very successfully after modification of the viscosity. Part of the salad oil is replaced with hydrogenated vegetable oil and the total amount is reduced to about 40%.

Whipped butter, based on a mixture of butterfat and salad oil, is very good on cornbread or other soft, crumbly bread. Cream cheese makes a good base for a number of canapé spreads. Chocolate and strawberry flavored toppings can be made using whipped cream, vegetable fat emulsions or marshmallow bases.

(*Continued on Page 73*)

TABLE II
WHIPPED CREAM STIFFNESS AND STABILITY

Cream Butter Fat Wt. %	*Propellant "Freon-C318" Wt. % in N_2O*	*Pressure psig Average*	*Stiffness (Force in grams) Time in Minutes* 0	1	2	3	4	5
30	0	102	63.5	50.0	47.8	45.5	44.0	43.0
30	15	98	73.0	66.5	63.0	61.5	60.0	58.5
30	20	98	73.0	59.0	55.0	53.0	51.5	50.0
30	25	102	70.0	57.0	54.5	51.0	50.0	49.0
30	30	100	74.5	62.0	58.0	55.5	54.5	53.5
20	0	103	66.1	51.3	48.9	44.2	42.0	41.0
20	15	95	72.0	62.0	60.0	59.0	57.0	56.0
20	20	91	71.5	61.0	59.0	58.0	57.0	56.0
20	25	100	76.5	64.0	61.0	60.0	59.0	58.0
20	30	[illegible]	76.0	64.5	61.5	60.0	59.0	58.5

Table III
WHIPPED CREAM OVERRUN AND DRAINAGE

Cream Butter Fat Wt. %	*Propellant "Freon-C318" Wt. % in N_2O*	*Pressure psig Average*	*Overrun % Average*	*Drainage ml/100 g. Average*
30	0	101	225	4.07
30	15	96	285	3.75
30	20	96	285	3.70
30	25	100	285	3.90
30	30	103	285	2.80
20	0	104	239	10.20
20	15	95	257	8.6
20	20	101	257	8.3
20	25	100	272	7.8
20	30	97	257	8.5

N_2O and "Freon C318"

(*From Page* 22)

These aerosol food products have been made by several laboratory formulations, using straight nitrous oxide, straight "Freon-C318" or an appropriate mixture as propellant. Just as in the nonfood aerosol product line, there is an optimum propellant mixture for each formulation of the product. In some cases, a mixture of nitrous oxide and "Freon-C318" is better than either propellant alone. Where outstanding stability and firm consistency are required for a foam, straight "Freon-C318" give the best results.

It is still premature to discuss costs of aerosol food products containing "Freon-C318" propellant. Many details of product formulation and production remain to be worked out. Commercial availability of "Freon-C318" and of its mixtures with nitrous oxide is still several months away. But there are some promising indications.

A regular 211 x 413 aerosol can, which holds about 7 ounces of conventional whipped cream, can hold about 13 ounces of cream with straight "Freon-C318." The unit cost per container is higher with "Freon-C318" — about 40 cents versus 23 cents for the nitrous oxide package. But the higher net weight means a saving of packaging costs per pound of food product, which should be important to the consumer if it is explained to him. For typical formulations, the costs per pound of cream are about 50 cents for the package with "Freon-C318," and about 52 cents for the nitrous oxide package. This means that a better product can be made at less cost to the consumer by using "Freon-C318."

Using a mixture of "Freon-C318" and nitrous oxide would add about half a cent to the cost of a 7-ounce package. As shown above, markedly better quality whipped cream is produced by the mixture versus straight nitrous oxide. The cost can be reduced, with little or no drop in quality, by reducing the butterfat content of the cream used with the mixed propellant.

Aerosol whipped cream sales have climbed from nothing in 1948 to about $100 million last year. Total food aerosol sales will climb rapidly in the next few years as this new propellant mixture is used to develop and promote more aerosol food products of improved quality.★

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A method of measuring nitrous oxide, nitrogen and oxygen in the blood

J.B. BOWES, MB, BS, FFARCS

Department of Anesthesiology
University of Washington Medical School
Seattle, USA

This paper describes a method of measuring the tensions of the gases nitrous oxide, nitrogen and oxygen in the blood, by the application of gas chromatography to analyse the gas contents of a small bubble which has been equilibrated with those gases which are dissolved in the blood.

In 1907 August Krogh[1] defined the tension of a gas in a fluid as the partial pressure of the said gas which is in diffusion–equilibrium with the fluid. So that from this definition the 'direct' way of determining the tensions of a mixture of gases dissolved in a fluid is to equilibrate a volume of air with this fluid. This equilibration occurs across the fluid gas interface by the net transfer of molecules of a gas from regions where the pressure exerted by these molecules is high to regions where this pressure is low. After a time the number of molecules of each gas crossing from liquid to gas is equal to the number crossing from gas to liquid and then the 'air' is said to be equilibrated and its composition has changed to that of the composition of the gases in the liquid. As the percentage volume of each gas of the total volume of the mixture of gases is equal to the percentage pressure of each gas of the total pressure exerted by the gases[2]; which since they are in equilibrium with the ambient atmospheric pressure means that each gas can be expressed as a percentage of the ambient atmospheric pressure. So that the percentage composition by volume of the gases in the equilibrated 'air' measures the percentage partial pressure which these gases exert in the liquid. If the volume of the gas is small in relation to the volume of the fluid sample then in this process of equilibration little gas, relative to the volume dissolved in the fluid will be transferred from the liquid to the gas phase, so that there will be little change in the tensions of the gases in the fluid: also with a small volume of gas, such as a bubble, where the surface area of the interface between gas and blood is large in relation to the volume of

the gas sample the time for equilibration of gases between fluid and gas will be short. Krogh[1] measured the gas tensions in a fluid by determining the percentages of the gases in the bubble by a micro-absorption technique. That is the individual constituents of the bubble were selectively absorbed by chemical reagents, and the resulting decrease of volume of the bubble was measured by the decrease in length which the bubble occupied in a capillary tube.

Before Krogh's experiments[3,4], measurements of blood gas tensions had been performed by analysing a larger volume of air which had been equilibrated against blood *in vivo*. This was done by passing the blood from an arterio-venous shunt over a long cylinder which was contained within another cylinder of a slightly larger diameter so that the air inside this larger cylinder was thus exposed to a large surface of blood to produce equilibration of the gases. Since large volumes of blood were used this method was therefore deemed to be suitable only for animals. After Krogh's work the direct method of determining blood gas tensions was developed[5] and defined by Riley *et al.*[6,7] so that a bubble of air of approximately 10 micro-litres was equilibrated in a Roughton-Scholander syringe[8]: that is a syringe which has the glass capillary fused to the nozzle so that equilibration and analysis of the bubble are performed in the same piece of apparatus. During these manipulations of the micro-absorption technique the syringe is kept in a water bath at 37° Centigrade. Greater accuracy of analysis was achieved by increasing the size of the sample and the length of the capillary[9] or by halving the diameter[10] of the capillary tube. Other workers[11,12] transferred the equilibrated bubble into a Scholander apparatus for the micro-analysis by the method of Scholander[13]. These micro-absorption techniques are technically difficult, require constant practice and are time consuming. As nitrous oxide is appreciably soluble in the weak solutions of sodium hydroxide used in the absorption of the carbon dioxide readings for both of these gases would be inaccurate. Several authors[14,15,16] have pointed out that nitrous oxide is hardly soluble in concentrated solutions of potassium hydroxide but this is such a viscous liquid that Nunn[17] found the manipulations of the bubble and blood necessary in the analysis extremely difficult. Thus the failure to develop the direct way of measuring blood gas tensions in the presence of nitrous oxide is due to the difficulties of the micro-absorption of the bubble rather than the equilibration. Though Strang[18] successfully used the mass spectrograph to measure the contents of the equilibrated bubble, and this method has the advantages of small sample size and simplicity of use, it has not been used to measure nitrous oxide tensions and the initial high cost of the apparatus and difficult maintenance are disadvantages which will probably prevent its widespread use.

As the direct determination of nitrous oxide tension in the blood has been difficult methods of measuring it in the blood have been measurements of 'content' and since this gas in solution follows Henry's law[19] the change to tension has been easy. Though the content of nitrous oxide in the blood could not be determined by the manometric method of gas analysis originally described by Van Slyke[20,21], modifications of this method by the application of correction factors[22], the content of the nitrous oxide could be determined after the absorption of the oxygen and the carbon dioxide. These correction factors are necessary because a part of the nitrous oxide released in the vacuum extraction is reabsorbed by the sample and the reagents due to the reapplication of the pressure when the volume of the nitrous oxide is measured. These correction factors depend upon the Ostwald solubility coefficients and these can also be determined by the Van Slyke manometric apparatus[23]. Kety[24] simplified the measurement of nitrous oxide content by absorbing both carbon dioxide and oxygen with a single reagent and ignoring the nitrogen which due to its low solubility in the blood would have a small content in spite of a high tension. Also after vacuum extraction Lawther and Bates[25] measured the nitrous oxide content of the blood by determining the percentage of the nitrous oxide in the released gases against a background of oxygen by a Luft type of infra-red analyser.

Gas chromotography was applied to the measurement of blood gas tensions in the hope of simplifying the analysis and of obtaining the results for oxygen, nitrogen, nitrous oxide and carbon dioxide by a single simple manoeuvre. Gas chromatography is a versatile and cheap way of measuring gases and vapours, and this technique has been applied to the measurement of anæsthetic gases and vapours by Hill[26,27] and others[28]. Butler *et al.*[29,30] and other workers[31,32,33,34] describe its use in the determination of anæsthetic gases and vapour in the blood, while the use for the determination of the respiratory gases in the blood has also been described[35,36,37,38,39].

Gas chromatography will only measure gases and vapours in the gas phase, thus in the measurement of gases in the liquid phase the gases have to be extracted first. This has been done by manometric vacuum extraction[35,37,39] which has the advantage of simplicity. But the disadvantages are that it requires a certain amount of skill to make certain that the volume and pressure of the sample of gases to be analysed after the vacuum extraction is constant. With the gases which are soluble in the blood or reagents such as carbon dioxide or nitrous oxide, the level of the mercury should be returned to the original level at a fixed rate so as to standardize the volume of nitrous oxide or carbon dioxide which is reabsorbed[31].

Other authors have extracted the dissolved gases by injection of

a disk of sintered glass through which the carrier gas was flowing. My limited experience of this technique suggested that the sample of gases came off too slowly so that the signals were difficult to interpret, and that the extraction of the blood sample gases was not complete. Moreover the chamber into which the blood was injected soon became fouled so that after a few analyses part of the apparatus had to be dismantled. Other methods of extraction include the use of a solvent such as N heptane[29,30] which proved extremely satisfactory for vapours and distillation[33].

METHOD

A 2ml sample of blood is required for the analysis and this is drawn into a 2ml syringe, which has a plunger with a cone shaped rubber end which fits into a recessed end of the barrel. This syringe was specially made by filling the nozzle end of the syringe with perspex cement and drilling with a cone shaped drill. The reason for this shape of syringe is to prevent the bubble from being trapped in the shoulder of the barrel surrounding the concentric nozzle of the syringe. Before the sample of blood is drawn the dead space of the syringe is filled with a 10mg/ml solution of heparin. This prepared syringe is used to take the 2ml sample through a fourway tap (the bubble introducing tap) which has two bores each of 20 microlitres in volume set at right angles to each other in the body of the tap. These bores run straight through the tap arching over each other with no internal connection between them. As these two bores are at the same level, a turn of 90° reverses the bores in relation to the limbs of the tap; this means that during the filling of the syringe with the blood sample a turn of 90° of the tap will introduce the contents of the other bore into the syringe and by this means a bubble of standard volume can be introduced into the blood sample. The gas which is passed through the limbs to produce this bubble can be end tidal expiration, gas from a specially prepared cylinder, or air. It is an advantage that the percentage composition of this gas does not differ widely from the percentages of gases dissolved in the blood sample.

The syringe which now contains 2ml of blood and heparin together with the 20 microlitre bubble is sealed by a cap and attached to one of the clips on a drum which revolves at 45rpm in a Grant Cambridge water bath set at 37°C. This temperature is checked by a thermometer immersed in the bath to be ±0.1°. The attachment of the syringe is at an angle to the axis of rotation so that during rotation the syringe not only revolves through the path of a circle but also the nozzle of the syringe oscillates up and down so that the bubble in the syringe travels through an elongated spiral from one end of the syringe

to the other so that the surface of the bubble is brought into contact with as large a portion of the blood sample as possible. This equilibration of blood and bubble lasts for 6 minutes.

After this period of equilibration the syringe is attached to one of the limbs of the second of the fourway taps (the sample introducing tap) which is of the same design as the bubble introducing tap except that the volume of each bore of the tap is 15 rather than 20 micro-litres. The limbs and barrel of this tap are made of perspex so that the interface between the blood and the bubble can be seen. The limb opposite to this limb of the syringe attachment is open to the atmosphere with a container for the waste blood. This means that the contents of the syringe including the bubble will be at atmospheric pressure in the tap. Through the other two limbs and bores of this tap the carrier gas of the gas chromatograph is flowing continuously.

As a pressure of 12lb/square inch is necessary to drive the carrier gas through the apparatus the sample introducing tap is greased with high vacuum silicone grease to keep it free from leaks. In spite of careful precautions some of the grease might contaminate the bores of the tap and to prevent this grease absorbing some of the gases in the equilibrated bubble a part of the sample of the blood is injected through the limbs and bores of the tap. By this passage of blood the grease and surfaces of the glass are brought to the tensions of the blood so that when the equilibrated bubble is manipulated through the tubing of the tap there is no alteration of the bubble composition due to re-equilibration with these surfaces. Aided by the cone shaped recess of the syringe the bubble is easily and cleanly expelled from the syringe into the polythene tubing connecting the syringe with the tap and then manipulated along the tubing until the bubble occupies the bore of the tap.

As the tap, syringe and tubing are in the same water bath as the drum for equilibrating the syringes the temperature is constant, and providing that the tensions of the individual gases in the blood do not differ widely from the tensions of the mixture of gases initially used for the bubble, or that the sum of the tensions of the gases dissolved in the blood sample is equal to the ambient atmospheric pressure there will be no change in the volume of the bubble during equilibration. This means that the 20 micro-litre bubble will be slightly larger than the bore of the tap so that a little of the bubble will occupy a small part of the limbs of the tap adjacent to the bore; this will make certain that there is no blood in the bore of the tap. Thus a turn of 90° reverses the bores so that the bubble sample is introduced into the apparatus and is swept by the carrier gas in to the gas chromatograph. Figure 1 shows diagramatically the equilibration and figure 2 shows a block diagram of the apparatus.

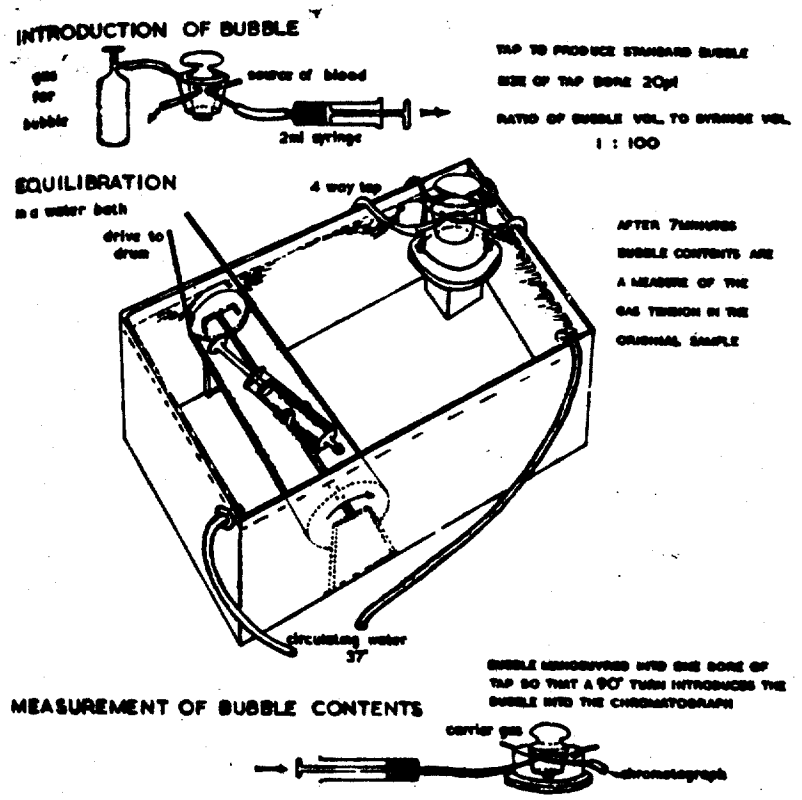

FIGURE 1 Diagram showing the steps in equilibration of the bubble and the measurement

Gas chromatography is a physical method of separating a mixture of gases. Figure 3 shows that a gas chromatograph is composed of five parts and that the separation is achieved by the individual components of the mixture of gases taking different lengths of time to travel through the apparatus. The individual gas is qualified by the interval of time from injection and quantified by the response of a non-specific physical detector of gases such as a thermistor which measures change of specific heat of the gas against the steadily flowing carrier gas.

In the gas chromatograph used in these estimations the carrier gas or mobile phase is hydrogen, reduced by an 'Endurance' valve and the pressure fluctuations are smoothed by a Negretti precision valve. So as to provide a baseline for the detector unit this carrier gas is passed around two of the four thermistors used in the detection of the gases

BLOCK DIAGRAM

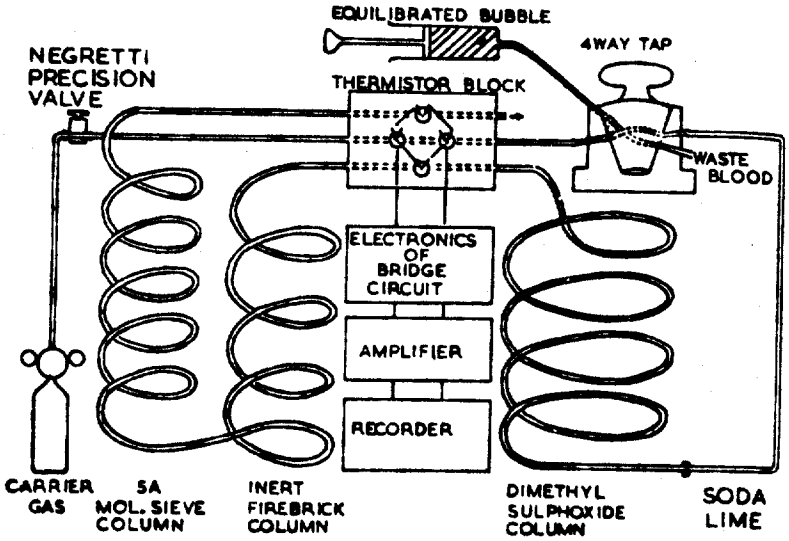

FIGURE 2 Block diagram of the apparatus showing the relationship of the parts

before passing through the bore of the sample introducing tap. These thermistors are the 'reference' thermistors. The sample introducing tap has already been described.

The third part of the gas chromatograph is the part which affects the separation and consists of two phases. The first is the mobile phase or carrier gas into which the sample is injected. The second is the stationary phase over which the sample is swept by the carrier gas or mobile phase. Because the different molecules of the mixture of gases in the sample which is to be analysed have different affinities for the material of this stationary phase, the components of this sample are differently distributed between the two phases. This means that in spite of the constant flow rate of the carrier gas the components of the mixture will take different lengths of time to travel through a length of tubing packed by this stationary phase. This length of tubing with the packing of the stationary phase is known as a column.

Gas chromatography is usually divided into two groups depending upon the nature of the static or stationary phase; thus for a solid stationary phase it is gas–solid or absorption chromatography while for the liquid phase it is gas–liquid or partion chromatography. This division is not only one of material but is also of the mode of separa-

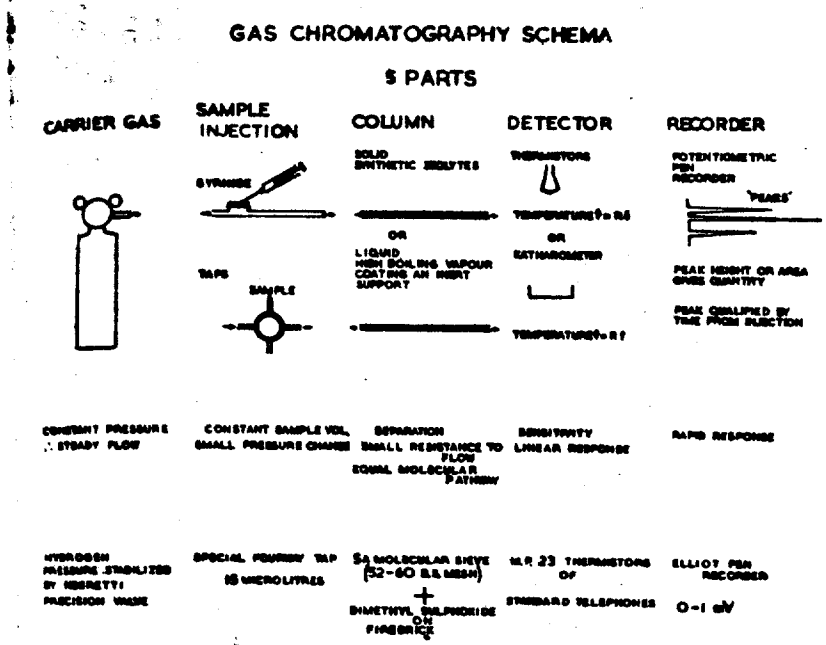

FIGURE 3 Schema of gas chromatography showing the five parts

tion, because in gas solid chromatography the packing material is a solid substance such as a synthetic zeolite called a molecular sieve where the water of crystallization has been driven off by heat. This leaves a cavity the size of which is accurately reproduceable for each type of molecular sieve, and it is these cavities which separate the components of the sample by differences of adsorption on the surface. This type of packing material is only suitable for separating gases of low boiling point as it permanently absorbs the gases of higher boiling point. With gas–liquid chromatography the material used to pack the column consists of a thin coating of a liquid of high boiling point with different solubility coefficients for the components to be separated, and this liquid is supported on an inert substance so as to make a suitable material for packing into the column. This type of column is suitable for separating mixtures of vapours or gases of high boiling point. Mixtures of gases of low boiling point pass through the column without separation. With the mixture of nitrous oxide, nitrogen and oxygen; the nitrous oxide will be separated from the other two gases by gas–liquid chromatography but the separation of nitrogen from oxygen will require gas–solid chromatography. Thus for this mixture of three gases it is necessary to have both types of column. In this apparatus the two columns are placed in series so that the sample size

can be kept as small as possible, though placing them in series does increase the difficulty of keeping the apparatus leak free; particularly the sample introducing tap.

The first of the columns is 22 feet (6.7 metres) of PTFE tubing of internal diameter $^1/_8$th of an inch (3.4mm) packed with 52–60BS mesh chromosorb which had been impregnated with 40% by weight of di-methyl sulphoxide. This column separates a mixture of nitrous oxide, nitrogen and oxygen into a component containing both nitrogen and oxygen and the nitrous oxide component. It was hoped to use this column to analyse carbon dioxide as well but the signal from this gas followed so soon after the nitrous oxide component that in the proportions of these gases which would be expected during anæsthesia the large nitrous oxide component which immediately preceded the carbon dioxide component caused such inaccuracy that this plan was not pursued. It was also thought that the measurement of carbon dioxide would be even less accurate if the nitrous oxide was varying considerably. For this reason between the sample injection tap and the first column a 9 inch (23cms) column of PTFE tubing of internal diameter $^1/_8$th of an inch (3.4mm) was placed, this packed with soda lime which had been crushed and sieved to be within 52–60BS mesh. This material as well as absorbing the carbon dioxide also decreases the dead space of the apparatus which is so necessary after the sample injection, so as to preserve a sharp signal. Also this column provides a small expendable column so that the accidental injection of blood will not affect the longer and less accessible columns. After the di-methyl sulphoxide column the first of the detector thermistors will produce a signal corresponding to the component of nitrogen and oxygen followed by a signal from the nitrous oxide.

Before the passage through the second of the separating columns the sample of gases is swept through an inert column; that is a column which has no power to separate the mixture of gases. In this case the column is 20 feet (6.1 metres) of copper tubing of internal diameter $^1/_8$th of an inch (3.4mm) packed with 52–60BS mesh chromosorb. This inert column delays the signal from the second detecting thermistor so that there is no overlap of signal from the two detecting thermistors. The sample then flows through the second column which is 20 feet (6.1 metres) of the same copper tubing as the inert column and packed with 52–60 mesh 5A molecular sieve. The copper tubing is necessary to keep the molecular sieve absolutely dry, for any moisture which could pass through the walls of the column would be absorbed as water of crystallization for the molecular sieve, which would inactivate it as a separating material. This column permanently absorbs the nitrous oxide but separates the nitrogen and oxygen so that from the second detecting thermistor placed at the end of this column there will

be signals for these two gases of low boiling point. From the second detecting thermistor the carrier gas elutes to the atmosphere at 30ml a minute.

The fourth part, the detector part, of the gas chromatograph consists of four thermistors embedded in a block of stainless steel so as to prevent sudden changes of temperature either from each other or with the ambient atmospheric temperature and for the same reason the columns and the electronics are placed in a small box. As there is no form of heating these columns are assumed to operate at 20°C. This lack of thermostatic control makes for simplicity but it means that the apparatus requires at least two hours of operation before thermal equilibrium is reached, and even after this period there is some baseline drift, but recalibration has not shown any change of amplification. The thermistors are the M.P.23 of Standard Telephones. Each one is operated at 10 milliwatts and the four are set in two limbs of a Wheatstone bridge, which is supplied by 10.4 volts D.C., stabilized across a Zenner Diode OAZ 212, after rectification by a solid rectifier circuit from the 230 A.C. volts mains supply. This stabilized voltage is also used for the amplifier circuit. This form of voltage stabilization was found on the initial experiments using gases as samples to be very satisfactory, but with the addition of the water bath for the blood bubble equilibration it was found insufficient to prevent fluctuation of the voltage, as the heater element 1.1 k Watts in the water bath was switched on and off. This fluctuation produced a signal which appeared as an unidentifiable gas, and later a further stabilized voltage

CIRCUIT DIAGRAM

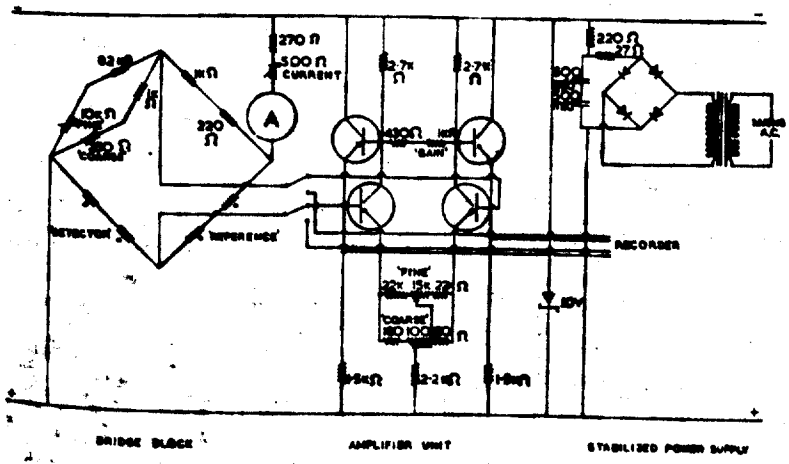

FIGURE 4 Full electronic circuit of the apparatus

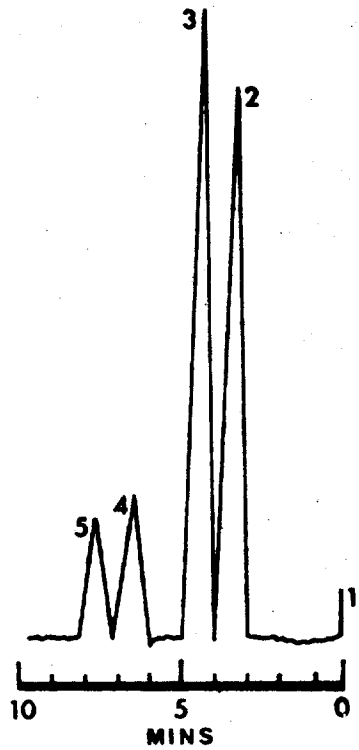

FIGURE 5 Typical chromatogram showing the peaks for the gases

transformer was used as a supply for both chromatograph and recorder. The signal is taken across the two limbs of the bridge a. fed into a differential amplifier circuit with a gain of 4, a high inp impedance and output impedance of 400 ohms. At a higher outpu impedance the potentiometric recorder fails to work. The full electronic circuit is shown in figure 4.

The fifth part of gas chromatography is the record and in this case the signal is displayed on a 0–1mV Elliott Potentiometric Recorder with a six inch scale as a 'peak' from the base line and a typical chromatogram is shown in figure 5. Strictly it is the area enclosed by the 'peak' which corresponds to the amount of gas but in these investigations the simpler 'peak' height was used.

CALIBRATION AND ASSESSMENT OF RESULTS

The apparatus is calibrated against a mixture of the three gases prepared to known proportions of composition by the pressure dilution

method of Hill[40]. A Beckman D.2 paramagnetic oxygen analyser was used to check the percentage of oxygen in the mixture and it was assumed that if this percentage was correct that the other two gases were also correct. This calibrating mixture of gases was first of all humidified at body temperature by passing them through a bubbler immersed in the water bath. The gases (BTPS) were then passed through the sample limb of the sample introduction tap. The injection of this sample into the gas chromatograph will produce peaks on the record so that a calibration chart can be made for each gas. The easy way to calibrate is to compare the peak of each gas with the calibration line between the two known peaks and so assume that the volume of each sample injected is equal, and due to the tap method of injection this is nearly so. Added accuracy is obtained by calculating the proportions of the gases one from another. In practice as the calibration is so easy the gases from two or more cylinders of different proportions are introduced before each run. As there is the possibility that the two bores of the body of the tap have not the same volume due to the contamination of one or both bores of the tap with the grease of the tap it is essential to pass at least two consecutive samples of the same mixture of gases.

A 'Blank' reading is obtained by passing hydrogen which has been saturated with water vapour at body temperature through the sample injection tap. With the introduction of this blank sample there is an immediate 'peak' followed by a swing below the baseline which corresponds to changes of the carrier gas pressure on the thermistors. So sensitive are these thermistors to changes of pressure that the addition of a bubble flow meter to measure the flow rate at the outlet of the apparatus causes noise on the record due to the small fluctuations of pressure from the formation and breakdown of the bubbles. These pressure peaks of the sample introduction are over before the peaks which correspond to any of the gas components are recorded, but these pressure peaks could confuse the record if a second sample was introduced into the apparatus whilst the gas peaks from the previous sample were being recorded.

The apparatus was assessed by measuring the tensions of gases in the blood which had been tonometered in the assembly described by Thornton and Nunn[41] where the gas mixture of known composition by pressure dilution is humidified at body temperature and passed at about 100ml a minute through a 200ml tonometer revolving at 30rpm. The end of the tonometer opposite to the end through which the gas is flowing is open to the atmosphere so that the pressure in the tonometer is that of the ambient atmosphere. Through this open end a 25ml sample of freshly drawn citrated pig's blood is introduced and equilibrated for at least half an hour against different mixtures of the

three gases within the range 5–80% for nitrogen, 10–80% for nitrous oxide and 5–20% for oxygen: no attempts were made to test oxygen at higher percentages because all equilibration methods are inaccurate at partial pressures above those found in the normal atmosphere[18,41]. The ranges of gases chosen were those that were expected in the clinical investigations which interested the author. 2ml samples were taken from this tonometered blood via a long nylon catheter so that the sample would be taken from the end of the tonometer into which the mixture of calibrating gases was entering and furthest from possible atmospheric contamination. The end of the catheter was kept below the surface of the film of blood by the weight of a small bevelled tip of copper and the other end was attached to the bubble introducing tap which was situated below the projection of the level of the surface of the blood in the tonometer. When the blood had been drawn to the outlet of the tap the flow of blood was maintained by syphonage. Since the dead space of the syringe had been filled with 10 mg per ml of heparin an anerobic sample could be obtained. Before the 2ml of blood had been completely drawn into the syringe the bubble introducing tap was turned through 90° so that the gas contents of the other bore were introduced into the syringe together with the 2ml of blood. The gas used to flush through this bore and so form the initial mixture of the bubble was air, if the tensions of the gases dissolved in the blood sample resembled the partial pressures of the constituents of air. Otherwise this mixture of gases was provided by rotameters which were set to deliver roughly the same proportions as were dissolved in the blood. The syringe was sealed, equilibrated and the bubble analysed as previously described. The signal peaks on the recorder were calibrated against peaks produced by a known mixture of gases[40]. Twenty–four consecutive investigations were performed and the coefficient of variation for nitrous oxide was 0.9% and that for oxygen and nitrogen was 2.0%.

DISCUSSION

Anæsthetists have been interested in tensions of the gases which they have administered to patients since 1876 when Paul Bert[43] administered nitrous oxide at pressures above atmospheric pressure in an attempt to increase the potency of this anæsthetic. More recently Butler[44] clearly reasoned that control of the brain tension and hence degree of anæsthesia is through the arterial tension of the anæsthetic so that the measurement of the tension of nitrous oxide in arterial blood is of great interest to anæsthetists. This method described for analysing the three gases nitrous oxide, nitrogen and oxygen produces a result within a quarter of an hour; six minutes for the equilibration

and about five minutes for the result to come through the apparatus. The volume of blood required for each measurement is 2ml.

The advantages of chromatography are its versatility, cheapness, ease of maintenance and the lack of technical skill required in the manipulation of it. In the method described here for the measurement of a bubble which has been equilibrated against blood-containing anæsthetic agents, various gases such as xenon could easily be measured by a change of packing material in the column. For the successful measurement of anæsthetic vapours such as halothane an addition of a thermostatically controlled oven is required, as the liquids which are used to separate these vapours function better at temperatures around 30°[30]. Once this addition has been made then the apparatus can with easy modifications of the columns be made to analyse any anæsthetic mixture, though due to the much higher solubility of the anæsthetic vapours in the blood, the equilibration of the blood to gas would not be nearly as critical and the volume of gas to blood could be far less than the 100 to 1.

The chief difficulty in the use of this apparatus is preventing leaks in the sample introducing tap. This means that the apparatus cannot be left on over night so that there is the possible problem of baseline shift. The leak free operation of this tap depends upon obsessional cleaning, drying and greasing. A leak not only produces a shifting baseline as the effects tend to speed the flow of carrier gas past the reference thermistors and slows this flow past the detector thermistors, but it also results in a change of calibration, as the leak will cause a decrease in the volume of the sample. The only technical skill required in the use of this apparatus is in the positioning of the bubble astride the bore of the tap. Stops on the tap at 90° apart help to make certain that the turning of the tap through this angle is accurate, fast and easy.

The inaccuracies of this method of measurement are due to incomplete equilibrium of the bubble with the blood; to inaccurate analysis of the bubble by the chromatograph and to the errors arising from the measurement of the record. The errors due to the incomplete equilibration of the bubble with the blood have been analysed by Shepard and Meier[45]. These errors are due to either imperfect equilibration or to change of tension of the gases in the blood during equilibration. Imperfect equilibration means that the tensions of the gases dissolved in the blood at the end of equilibration are not the same as the partial pressures of these gases in the bubble.

Riley *et al.*[7] equilibrated the bubble for five minutes and assumed that equilibrium had been reached at the end of this time. In the case of the more soluble gases this is so, but with nitrogen this is not so unless the tension of this gas in the blood is similar to the partial pressure in the bubble.

Another cause of failure to reach equilibrium is due to the sum of the tensions of the gases dissolved in the blood differing from the ambient atmospheric pressure. If there is a difference this will set up a condition of 'sliding equilibrium' which was first described by Krogh in 1907[1], but has not always been appreciated since then. For example if the sum of the partial pressures of the gases in the liquid phase is higher than the atmospheric pressure, then the volume of the bubble will incessantly increase. Some workers [12,46] found that the sum of the tensions of the gases dissolved in the venous blood and those dissolved in arterial blood was below and approximately equal to the ambient atmospheric pressure respectively. This factor therefore will not lead to error in the measurement of blood gas tensions in the arterial blood.

The other cause of error at equilibration is the alteration of the tensions of the dissolved gas in the blood which occurs during equilibration: for example from the change of tension of a component due to the transference of gas from one phase to another. Riley *et al.*[6] showed that with a bubble of about 15 micro-litres and a blood to bubble ratio of 100 to 1 the tensions of the gases would not alter by more than 1mm of partial pressure. Due to the nature of oxygen dissociation in the blood, at high oxygen tensions a small change of oxygen content will produce a relatively large change of oxygen tension, so that equilibration changes will lead to greater inaccuracies at high partial pressures of oxygen than at low pressures.

Also during the equilibration there will be metabolic changes due to the respiration of the red blood cells. These metabolic changes were shown by Wiesinger and Saboz[47] to depend upon the leucocyte count and in blood containing 10,000 leucocytes per cubic mm the oxygen saturation would fall by 0.25% of saturation. This again would have greater influence when the oxygen tension of the sample was high, and due to these reasons Nunn[42] stated that equilibration methods of measuring oxygen tensions above 95mm of mercury are too inaccurate to be useful, and certainly it does not appear that this application of chromatography has anything to offer to rival the polarograph in the measurement of oxygen tension. Another possible cause of loss of dissolved gas in the sample is through the interface between the barrel and the plunger of the syringe into the water bath.

The gas chromatograph itself is responsible for the difference in the coefficient of variation between the nitrous oxide and the other two gases, because there is absorption of part of the low boiling gas component so that after the two columns the sum of the oxygen and the nitrogen 'peaks' is only about half the height of the composite peak. Also the shape of the nitrous oxide peak is narrower and sharper, due partly to the shorter total pathway for the molecules of the sample to

travel, but also as Hill[27] mentions, gas–liquid produces a better 'peak' than gas–solid chromatography.

The apparatus has only been used by the author and during clinical use has given very little trouble.

Acknowledgements

Thanks are due to the late Professor Woolmer for his encouragement and for providing the facilities. Drs Butler, Hill, Nunn and Strang gave much helpful advice. Mr R. Stone designed the amplifier unit and Mr S. R. Mable, Mr J. Hook and Mr S. Askill patiently gave much needed technical assistance, while Mr S. H. Redman took the photographs.

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EXPERIMENTS WITH ANESTHETIC GASES

W. EASSON BROWN AND VELYIEN E. HENDERSON

Department of Pharmacology, University of Toronto

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A little over a year ago we reported on some experiments with propylene (1) as an anesthetic which showed that it was an anesthetic gas with a higher potency than any heretofore studied. We found, however, that at present it could not be obtained commercially and as we were not able to compress it we were handicapped in its further study. It had, like ethylene which we had previously described, a somewhat disagreeable odor and was, like it, explosive if mixed with air. Like ethylene it contains a double bond, $CH_2 = CH \cdot - CH_3$, and although it would be considered by chemists a relatively inert gas still both propylene and ethylene can hardly be looked on as being as inert as nitrous oxide which is only acted upon by other chemical substances with great difficulty.

It appears that the ideal anesthetic gas should be one with a sufficient solubility in water to be carried to the central nervous system and such a high degree of solubility in the lipoids as to lead to its differential accumulation there. Further, such an ideal anesthetic should leave the body promptly and entirely unchanged. In the latter respect nitrous oxide amongst the older anesthetics was the best. It has, however, the great disadvantage that its differential solubility in fat as compared with water is so low that it only accumulates in the central nervous system to a sufficient concentration if the partial pressure in the inspired air is so high that anoxemia is present. The changes which it produces in CO_2 and pH combining power are due to the anoxemia (3).

It therefore seemed that before making a more detailed examination of the effects of propylene as an anesthetic it would

be wise to ascertain whether some other gas free from double bond and odor and highly inert chemically would produce a satisfactory anesthesia.

This investigation was the more alluring in that some preliminary experiments with methane had led to the conclusion that it acted as an anesthetic in a concentration of some 90 or 95 per cent with oxygen. We now believe that we were deceived in our experiments which were we realized too few in number to do more than show that it was not very promising. As the anesthetic potency of the alcohols is known to increase as the series is ascended, and as propylene was more anesthetic than ethylene, it was natural to suppose that the gases of the methane series would also increase. We chose propane as the first member to be studied.

This gas was made for us by Mr. L. J. Bonham of this laboratory by Grignard reaction. This yielded a gas which after washing and drying was exceptionally pure, containing some 97 per cent propane. Our thanks are due to Mr. Bonham for the preparation of this gas and the others used by us and also for much help during our experiments.

In order to conserve the gas, which is expensive to prepare in this way, we employed the following procedure: A cat was quickly anesthetized with a high percentage of ether, the trachea opened and a three-way cannula inserted. The carotid was prepared for blood pressure connections, the wounds bathed with novocaine and the animal connected by a clipped-off rubber tube to a cannister containing soda lime which in turn was connected to a large water-sealed volume recorder with a capacity of about one litre. Cannister and recorder were filled with an anesthetic mixture of oxygen and gas, the recorder only to a minimum volume sufficient to allow of respiration. The connections were as large and short as possible. The animal was allowed to breath through the third limb of the *T* till struggling occurred when most of the ether was eliminated, it was then made to breath the anesthetic mixture by connecting to the cannister and recorder.

It was found that even with percentages of propane as low

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as fifty, respirations sometimes stopped for one or two minutes. If disconnected and allowed to breath air for a minute or two and then reconnected, breathing occurred normally and persisted. In both cases the cat ceased struggling but from time to time would twitch its tail or move a leg. Cutting the skin with the scissors appeared to produce no response. The animal was, however, not relaxed and the pupils were usually large and sluggish conjunctival reflex was present. Stimulation of the femoral nerve usually produced slight struggling with a rise of blood-pressure and quickened and deepened respiration, but in some cases no struggling appeared. Blood-pressure remained at a slightly less than normal level but the tracing showed peculiar irregularities which suggested missed heart beats.

Higher percentages of gas were then tried and a mixture of 90 per cent appeared to produce anesthesia with relaxation and absence of conjunctival reflex, no struggling on nerve stimulation and little change in blood pressure or respiration. This severe test which we have employed throughout our work indicates a complete surgical anesthesia. The blood-pressure had fallen but not dangerously and the heart's action appeared no more irregular than before. It will, however, be seen from this that propane is not likely to prove to be a useful anesthetic as the percentages required for relaxation and true anesthesia are too high. The reasons for the cessation of struggling may be that propane has some analgesic effect.

Experiment 18. Propane-oxygen. Cat, 3 kgm. March 4, 1925

The induction of anesthesia was commenced at 10:46 with a mixture which was estimated to contain 60 per cent of propane. At 11:00 an analysis showed the mixture to contain 43 per cent propane, and as the cat was not anesthetized, propane was added to give a 62 per cent mixture. At 11:21 the cat was still showing movements though they appeared to be involuntary in character. At 12:08 the blood pressure had fallen to 100 mm. Hg from 150, and respiration had slowed slightly. At 12:10 there was very slight response to electrical stimulation of the femoral nerve. The oxygen in the mixture was slowly allowed to increase. Blood pressure improved to 125. The mixture now contains 43 per cent propane. This was increased in the next twenty-five minutes to 53 per cent. The blood pressure remained unchanged. Electrical stimulation always caused some movements. From time to time movements occurred without stimulation though the animal showed no reaction to cutting the skin of the abdomen. At 12:00 the mixture was increased to 65 per cent propane which caused a drop of blood pressure to 100 mm. Hg.

Experiment 21. Propane-oxygen. Cat, 6½ pounds

The induction of anesthesia was commenced with a mixture of 89 per cent propane. Blood pressure was 125 but showed a steady fall. After one minute's inhalations blood pressure was 55. At 10:03 (three minutes after induction began) stimulation caused slight movements. At 10:04 blood pressure was 30 and cat was allowed to breath air. At 10:12 cat put on a mixture of 82 per cent propane. Blood pressure was 115. At 10:16 blood pressure was 100 and stimulation caused some movement. This response to stimulation remained and at 10:33 blood pressure was 90. Propane was added to 87 per cent—in a minute and a half blood pressure was 65 and cat showing movements. Animal again put on air at 10:39. At 10:45 cat on mixture of 93 per cent propane, blood pressure 130. At 10:48 blood pressure fallen to 50. Animal again allowed to breathe air and at 10:58 was again put on 93 per cent propane. Blood pressure was 150 but fell rapidly. At 10:59 electrical stimulation caused considerable movement. At 10:59½ blood pressure was 80. Some oxygen was added and blood pressure rose to 100. At 11:04 stimulation caused some movement and respiratory increase. Blood pressure fell slowly and at 11:09 was 50. At 11:10 electrical stimulation caused no movements and but slight respiratory increase.

These protocols are typical of many others.

Higher members of this series have been studied by Fühner (2) and it was evident from his experiments that the anesthetic and toxic doses of pentane and heptane lay very close together. We also tried some pentane and also found that toxic and anesthetic doses were close together and that the action of the anesthetic was apparently uncertain because some secondary stimulation appeared to be produced which revealed itself in peculiar irregular movements, even when the anesthesia appeared to be complete.

This series did not give promise of producing a satisfactory anesthetic gas.

We also tried a few experiments with the next higher member of the propylene series, butylene. The butylene used by us was probably the ethyl-ethylene. We found that the percentage of butylene which would maintain anesthesia was about 30 per cent in cats. With even this percentage the blood pressure was considerably lower than normal while with 40 per cent the blood pressure was very low. The period of induction was relatively prolonged if just anesthetic percentages were used. There was a very marked stage of increased tonus and rigidity in some cases, especially in the hind limbs, though also apparent in the front ones and this led to a peculiar clonus such as one sees in decerebrate rigidity, especially in the type of decerebrate rigidity which occurs in animals with small doses of ether, and which is known to occur in some cases of ether anesthesia in man (ether tremor). We also found that this rigidity lasted for a long time, almost throughout a period of complete anesthesia, and that from time to time the hind limbs showed peculiar irregular movements which suggested a stimulation of the cord. This occurred even at a time when anesthesia was complete. This reaction was not so apparent when high percentages, 40 per cent or over, were used for induction. Butylene therefore does not appear to be as satisfactory an anesthetic as propylene.

Experiment 8. Butylene-oxygen. Cat, 7 pounds

The cat was induced at 2:15 with a mixture containing 60 per cent butylene. When anesthesia was complete the animal was connected with the respirometer containing a maintenance mixture of 49% butylene. This percentage was gradually lowered until at 2:45 the mixture contained 25 per cent butylene. The blood pressure was 110. This percentage gave a light degree of anesthesia, there being some response to electrical stimulation. This percentage was carried on until 3:29 without any apparent deepening of anesthesia, the blood pressure running at about 115, when the percentage of butylene was raised to 39 per cent. This percentage gave good anesthesia but however caused the blood pressure to fall to about 80. Respirations were regular throughout. The percentage of butylene was raised at

3:45 gradually until at 4:07 it was 50 per cent. The blood pressure had gradually fallen to 45. At 4:15 respirations ceased while the heart continued to beat. Three minutes artificial respiration caused the blood pressure to rise to 125 and respirations to be resumed.

Consequently it was evident that propylene appeared to be more satisfactory as an anesthetic having a wider range between anesthetic and toxic doses than any of the other gases that we had studied. We therefore undertook a study of the effects of propylene on metabolism and particularly on the changes in carbon dioxide combining power and hydrogen ion tension.

The work of Leake (3, 4) and his co-workers had shown that nitrous oxide caused changes in CO_2 combining power and pH leading to an alkali deficit. This may of course be secondary to the anoxemia which was present. He also reported that ethylene when anoxemia was present produced similar changes though not so marked in degree, while when absent, though there was a fall in CO_2 combining power and pH, these remained within normal limits.

Hence a similar study was made of propylene. Cats were used at first and they were anesthetized by breathing through a mask which absorbed CO_2 from a bag containing a propylene-oxygen mixture. As soon as possible they were connected by a tracheal cannula to the apparatus described above. It was found that at the end of forty minutes there was a slight change in CO_2 combining power which had increased on an average from 36 to 44 per cent and a fall in pH from 7.52 to 7.42. Both these figures fall within a normal metabolic range.

Experiment 17. Propylene-oxygen. Cat, 4¾ pounds

A preliminary blood sample showed CO_2CP 34.3 and pH 7.5. The animal was put on a mixture of 40 per cent propylene at 1:50 p.m. The cat soon became quiet, respirations were regular and blood pressure 125. The mixture was increased to 44 per cent. This gave a good anesthesia although there was some slight response to electrical stimulation. At 2:14 the blood pressure was 135. The animal remained anesthetized until 3:00 p.m. on a mixture containing from 44 per cent to 41 per cent propylene. The blood-pressure was at that time still 135. A blood sample at the conclusion of the anesthesia

showed CO_2CP 43.8 and pH 7.5. An analysis of the gas mixture showed it to contain propylene 42 per cent, oxygen 34 per cent, nitrogen 24 per cent.

Experiment 32. Propylene-oxygen. Cat, 6 pounds

A preliminary blood sample showed CO_2CP 27.7 and pH 7.5. At 2:45 the animal was induced with a mixture of 60 per cent propylene. Blood pressure at 2:47 was 150. At 2:52 electrical stimulation gave no response. The anesthetic mixture was changed by the addition of oxygen to 39 per cent. At 3:45 a blood sample showed CO_2CP 43.8, and pH 7.34. The blood pressure showed a very slow fall and at 3:45 was 135. At 4:04 the animal showed very slight response to electrical stimulation and the mixture was increased to propylene 50 per cent. At 4:33 a blood sample showed CO_2CP 43:8 and pH 7.32. The blood pressure was still 135. A gas analysis of the mixture showed it to contain propylene 46 per cent, oxygen 32 per cent, nitrogen 22 per cent.

Experiment 24. Propylene-oxygen. Cat, 6 pounds

Pre-anesthetic blood sample CO_2CP 39.9, pH 7.6. Cat was put on a mixture containing 60 per cent propylene. Good surgical anesthesia was maintained. At the end of one hour blood sample showed CO_2CP, 44.8, and pH 7.49, and in two hours 47.6 and 7.50.

We have repeated these experiments on dogs with a similar result, for example

Experiment 47

CO_2 combining power, 50.7 per cent, at the end of an hour 49.0. pH, 7.51, at the end of an hour 7.3. The blood sugar, 0.098 per cent, at the end of an hour 0.098 per cent.

This latter experiment shows a rather low pH but we have reason to believe that the soda lime absorbent for the CO_2 had become somewhat inefficient as the CO_2 percentage in the mixture was a little high at the end of the experiment. It is interesting to note that in 2 cases with cats where owing to a slip in technique the inspired mixture was rich in nitrogen when consequently there was a low percentage of oxygen in the mixture the CO_2 combining power fell in experiment 41 from 37.2 per cent to

27.7 per cent and the pH remained about the same 7.52 and 7.5. The oxygen in the mixture was 18 per cent, the propylene 55 per cent and the nitrogen 27 per cent. The changes in these cases are similar to those seen under ethylene with oxygen want. Leake and co-workers indicate the importance of more than the normal amount of oxygen in preventing changes in metabolism.

It may be noted that in the one case, experiment 50, on a dog there was no change in blood sugar at the end of an hour. This is the only case in which we have made sugar determinations as yet.

Propylene is fairly soluble in water 0.3 at 30°C. and consequently a large amount of gas will be absorbed by the water of the body, but the recovery is prompt. Dogs recover in ten to fifteen minutes and one dog has been anesthetized for over an hour on three occasions and is still quite lively and well and does not seem to dread the process.

Three of us have had short administrations of propylene sufficient to show that a 50 per cent mixture will probably suffice to induce anesthesia in man, though we have found that both dogs and especially cats required somewhat higher percentages to induce anesthesia rapidly.

In conclusion we would like to point out:

1. That the methane series will probably not yield an anesthetic gas.

2. That propylene appears a better anesthetic than ethylene, the percentage required being slightly more than half that needed with ethylene.

3. That owing to the large amount of oxygen that can be supplied there is evidence that the metabolic effects of anesthesia are decreased to a minimum with propylene.

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Current Perspectives

ANESTHETICS AS A CAUSE OF ABORTION

THOMAS H. CORBETT, M.D.

Anesthesia Service, United States Veterans' Administration Hospital, Ann Arbor, Michigan 48105 and University of Michigan Medical Center, Ann Arbor, Michigan 48104

The field of occupational health is not a new one. Probably the earliest publicized case of an occupational hazard was that of the English chimney sweeps who developed scrotal cancer from chronic exposure to soot. Other well-known cases of occupational disease include liver toxicity from chronic exposure to carbon tetrachloride in dry cleaning establishment workers, and more recently, development of various malignancies in workers due to chronic exposure to low concentrations of certain chemicals and dyes including bladder carcinoma due to benzidine exposure, and leukemia due to exposure to benzene.[1] Abortion has also been suggested as an occupational hazard due to exposure to X-rays.

Recent surveys suggest possible deleterious effects from chronic exposure to the operating room environment. Vaisman,[2] in 1967, reported an unusually high incidence of headache, fatigability, irritability, nausea, and pruritis among Russian anesthesiologists. He also noted that 18 of 31 pregnancies studied among female anesthesiologists ended in spontaneous abortion. In addition, two of these pregnancies ended in premature delivery and one in congenital malformation. Askrog[3] also noted an increased rate of spontaneous abortion among anesthetists in Denmark. Lencz and Nemes[4] conducted a survey of Hungarian anesthetists and female anesthesiologists. This study revealed that of 31 pregnancies among 24 individuals, 10 resulted in spontaneous abortion, 9 in pathologic pregnancies, and 2 in premature delivery. Only 10 deliveries were normal.

More recently, Cohen, Belville, and Brown,[5] in a controlled study, reported a 38% abortion rate among anesthetists and 30% abortion rate among operating room nurses, with a 10% abortion rate for a control group of general duty nurses.

This information suggests new parameters for the gynecologist to consider in the evaluation and treatment of the infertility patient. Although the number of infertile females working in operating rooms constitutes a small percentage of the total population of infertility patients, these surveys suggest that this group of patients may benefit by removal from the operating room environment if the operating room is not equipped with efficient gas-scavenging devices which are now available, but not generally in use. Possible etiologic factors for the increase in spontaneous abortion among operating room personnel include exposure to low concentrations of anesthetic gases and X-rays, exposure to viruses, exposure to chemicals and cleaning solutions used in operating rooms, fatigue from working long hours, and strenuous physical demands. Exposure to anesthetic gases is considered the most likely factor.

Although it has been 128 years since the discovery of inhalation anesthesia, it is only recently that attempts have been made to determine the concentrations of

anesthetic gases present in the operating room. In 1969, Linde and Bruce[6] reported levels of halothane averaging 10 p.p.m. and nitrous oxide 130 p.p.m. in the operating room, with peak concentrations as high as 27 p.p.m. halothane and 428 p.p.m. nitrous oxide.

Askrog[7] reported an average concentration of 85 p.p.m. halothane and 7000 p.p.m. nitrous oxide in the anesthesiologist's inhalational zone.

In 1971, Corbett and Ball[8] reported levels of methoxyflurane up to 10 p.p.m. in the operating room environment, and they noted detectable levels of methoxyflurane in end-expired air of anesthesiologists up to 30 hr. following exposure under usual operating room conditions. Subsequent studies by these investigators revealed operating room levels of halothane as high as 10 p.p.m. and detectable levels of halothane in end-expired air up to 64 hr. following routine occupational exposure (Fig. 1).

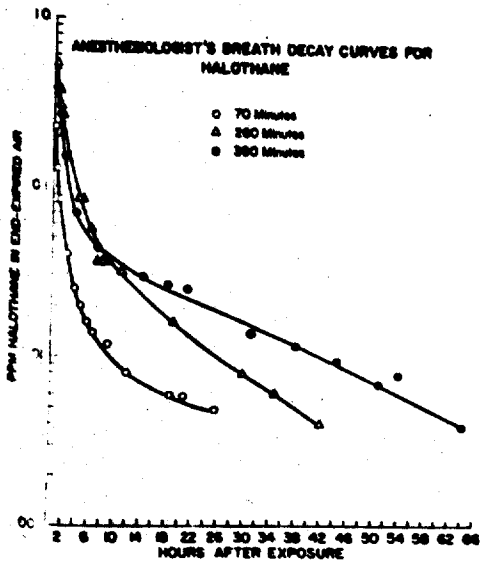

FIG. 1. Halothane breath decay curves in an anesthesiologist following exposures of 70, 260, and 390 min. Halothane was detectable in end-expired air for 26, 42, and 64 hr., respectively, following termination of exposure.

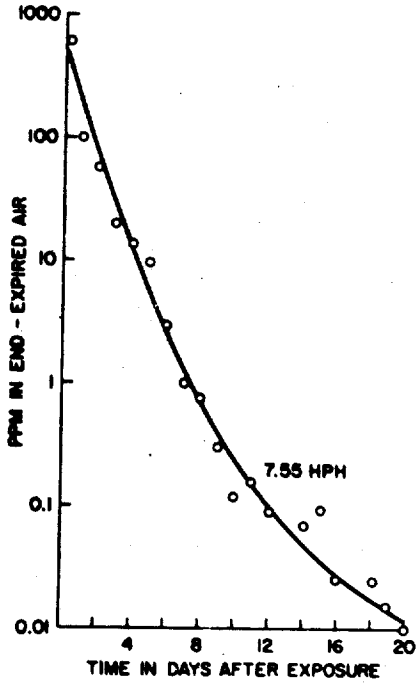

FIG. 2. Halothane breath decay curve for a patient following halothane anesthesia. HPH, 7.55, refers to the halothane-percent-hours, a time concentration parameter used to compare exposures. HPH, 7.55, is equivalent to receiving 1% halothane for 7.55 hr.

Further studies by these investigators revealed detectable levels of methoxyflurane in end-expired air of patients up to 18 days following methoxyflurane anesthesia and detectable levels of halothane in end-expired air of patients up to 20 days following halothane anesthesia (Fig. 2).

Similar studies with nitrous oxide revealed concentrations in the inhalational zone of the anesthesiologist ranging from 1000–3000 p.p.m. (0.1–0.3%), with peak concentrations as high as 9700 p.p.m. when the anesthesiologist and anesthesia machine were partially enclosed in a tent of surgical drapes. Nitrous oxide was measurable in end-expired air of anesthesiologists from 3–7 hr. following routine occupational exposure, and in patients up to 56 hr. following nitrous oxide anesthesia (Fig. 3).

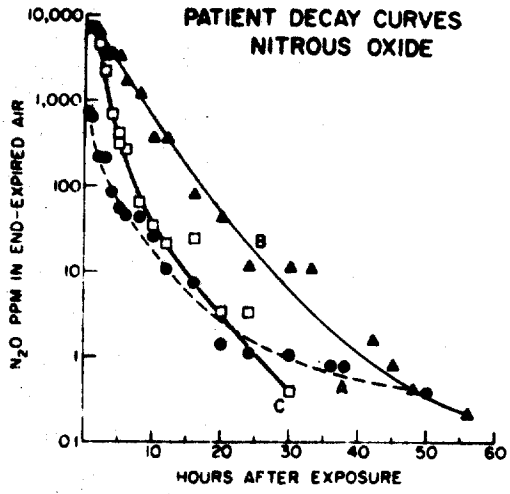

FIG. 3. Nitrous oxide breath decay curves for patients receiving nitrous oxide anesthesia for 30 min., 270 min., and 135 min. (Patients A, B, and C, respectively). Nitrous oxide was measurable up to 56 hr. following termination of anesthesia.

Stringent laws protect the industrial worker from exposure to numerous harmful chemical agents. Exposure to chemical vapors in industry is governed by Threshold Limit Values. No such limits have been set for most anesthetic agents, including halothane, methoxyflurane, and nitrous oxide.

At the present time, nitrous oxide is considered an "inert gas." However, several definite deleterious effects from chronic exposure to nitrous oxide have been established. Bone marrow depression in humans has been noted in several reports, and teratogenicity and embryotoxicity in rats at higher concentrations (50%) were reported by Fink, Shepard, and Blandau[9] and confirmed by Shepard and Fink.[10]

Biochemical mechanisms through which these deleterious effects manifest themselves are unknown. Studies of the possible metabolism of nitrous oxide seem imperative if we are to determine these mechanisms.

We have recently initiated studies in our laboratory to determine the effect of low concentrations of nitrous oxide on rat pregnancy. In the first part of the study, now completed, we have confirmed embryotoxicity of nitrous oxide in the rat when administered chronically, both at 1% concentration from Days 8–14, and at 0.1% concentration (1000 p.p.m.) administered from Days 12–20 of the pregnancy. Studies are in progress to determine the effect of intermittent exposure to these concentrations during the pregnancy.

We must now also question the safety of administering inhalation anesthesia to the pregnant patient. Will our anesthetic agents induce abortion or cause deformity of the fetus? We are unable to answer this question at the present time. There are no conclusive surveys to date which have explored this possibility. We have demonstrated nitrous oxide levels of 1000 p.p.m. in end-expired air of patients up to 10 hr. following anesthesia. We have demonstrated embryotoxicity of 1000 p.p.m. of nitrous oxide in rats. However, no amount of animal experimentation can define embryotoxicity, teratogenicity, or carcinogenicity in man. There is tremendous genus and species variation in the effect of drugs throughout the animal kingdom.

The gynecologist must extend the search for embryotoxic and teratogenic agents beyond the operating room. Numerous gases and volatile liquids have been introduced into the environment without adequate testing and have become part of the daily routine of modern women. The agents are present in hair sprays, spray deodorants, spray paints and other aerosols, cleaning agents and solvents, and in industrial plants which may employ pregnant women, to name just a few sources.

For the pregnant woman employed in an operating room environment, the proposed solution is relatively simple. She may either stay out of the operating room during her pregnancy, or work only in operating rooms equipped with efficient gas-scavenging devices, assuming abortions are caused only by exposure to anesthetic gases.

Another possible health hazard related

to operating room exposure was first suggested by Bruce, et al.,[11] in 1968, in a study on the cause of death among anesthesiologists over a 20-year period. This study suggested a higher than normal incidence of reticuloendothelial and lymphoid malignancies as the cause of death among anesthesiologists.

Chemists, who are also chronically exposed to low concentrations of volatile liquids and gases, have a similar history. Li, et al.[12] studied the cause of death among chemists and found a significantly higher proportion of deaths from cancer as compared to professional men in general. Nearly half the excess cancer deaths were attributed to malignant lymphomas and cancer of the pancreas.

Studies of chemical carcinogenesis have determined that certain chemical agents may not only be carcinogenic to the animal exposed, but also to its offspring. Certain agents administered to a specific species may exert lethal, teratogenic, or carcinogenic effects on the fetus depending on the dose of the agent and the stage of embryonic development during which the agent was administered. As yet, the possibilities of carcinogenic and transplacental carcinogenic properties of anesthetic agents have not been explored.

The discovery of toxicity from low concentrations of anesthetic gases should increase the stimulus for extensive research in the areas of metabolism and action of anesthetics, which in turn may provide new clues as to the mechanism of action producing embryotoxicity, teratogenicity, and possible carcinogenicity not only of anesthetic agents, but in the entire field of chemical carcinogenesis as well.

In conclusion, it is important for the gynecologist to consider environmental factors when evaluating the infertility patient and the patient with a history of repeated abortion. Does the patient work in an operating room or other area where she may be exposed to embryotoxic agents? Is she exposed to embryotoxic agents during the course of her daily routine? We must assume that nitrous oxide is only one of many as yet unidentified embryotoxins in our working and household environments. We must encourage further research to identify other agents and terminate exposure of pregnant women to them.

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STUDIES OF INTOXICATION

I. THE ACTION OF NITROUS OXIDE

BESSIE M. DAVIDSON

From the Pharmacological Laboratory, University of Aberdeen

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The fundamental ideas influencing these studies on intoxication were, (1) the investigation of the symptoms common to different types of substances in order to lay a basis for a syndrome of intoxication in general and, in particular, to determine the existence of any common conscious levels; (2) the determination of the differences, quantitative and qualitative, produced by such substances in order to aid in elucidating their mode of action; (3) the effect of intoxication on certain psychological problems affecting education. The last will be dealt with elsewhere. The methods employed were largely those of experimental psychology and the phases studied included states up to unconsciousness. Numerous studies of a similar nature under mild intoxication have been made with alcohol and other cerebral depressants, but none of these was adapted to the purposes in view. In the first place the action of these substances is too prolonged and they cannot be used for states bordering on the unconscious; and in the second place, owing to variations in absorption from the alimentary tract and the occurrence of excretion and in some cases of decomposition, the actual concentration in the blood for any particular syndrome could not be estimated, or, under the conditions of my experiments, be accurately determined by analysis. For the objects of the investigation a more labile form of administration and one which would afford a fairly accurate indication of the concentration of the substance in the blood was necessary. It was decided to employ compounds which could be administered by

inhalation and to use substances gaseous at ordinary temperatures. The vapour of volatile liquids was found to be unsatisfactory owing to condensation and consequent variation in the concentration of active gas inhaled. By employing gaseous mixtures, rapid alterations in the mental state could be induced and, since a constant partial pressure of the gas could be maintained in the lungs for a definite time, the effect of a definite concentration in the blood could be investigated. Further, in order to maintain the conditions as uniform as possible, one individual, who volunteered for the purpose, was used throughout the experiments. He is a healthy man somewhat over fifty years of age, of studious habit and good intelligence, with medical training and unaccustomed to alcohol or other narcotics. During the experiments, except for special purposes, he was seated on a chair. The gas mixtures were made for most experiments in Douglas bags of 135 liters capacity which were provided with the usual inlet and exit valves and nose clips. In the earliest investigations an ordinary face mask was used but even when considerable pressure was employed in its adjustment it seemed difficult to avoid dilution with air. For certain experiments a gas-bag capable of holding 1000 liters was employed. No re-breathing occurred in any of the experiments. Air, oxygen and nitrogen were used as diluent gases. The percentage of oxygen as well as the concentration of toxic gas is commonly given as it became evident with the progress of the research that this had an important bearing on some of the results. This will be seen in a later paper on the influence of anoxemia. Experiments were also made during recovery from inhalation of pure nitrous oxide gas. In these experiments the ordinary apparatus employed for inducing nitrous oxide anesthesia was used.

GENERAL EFFECTS

As an introduction to the experiments performed, the general effects induced by the different concentrations of nitrous oxide used, may be briefly described. The smallest concentration producing any subjective effect was 4 per cent which caused

six minutes after commencing the inhalation, a slight feeling of "lightness in the head." Ten and 20 per cent nitrous oxide caused a feeling of mild intoxication with paresthesia, and as will be shown later, some diminution in mental efficiency but induced no objective symptoms except inability to walk quite straight along a line. Otherwise balancing was apparently normal. Distinct objective symptoms were not observed until a concentration of 30 per cent nitrous oxide was inhaled. These were manifest in six minutes in slight muscular incoördination and lack of attention. With 40 per cent nitrous oxide the difficulty of concentrating the mind on the work to be performed became evident four minutes after the commencement of the inhalation. No excitement or increased talkativeness occurred with this concentration. These, with laughter, appeared when 50 per cent, was inhaled for three minutes. Later the speech became noisy and voluble. This concentration of nitrous oxide caused inability to hold the head up but otherwise while at rest muscular incoördination was not marked. It became very noticeable during the inhalation of 60 per cent and with this percentage the subject became aggressive and emotional. Under 65 per cent nitrous oxide, muscular incoördination became more marked and speech assumed a maudlin character. Unconsciousness, however, did not occur. This followed the inhalation of 70 per cent within six minutes and the inhalation of 75 per cent in approximately four and one-half minutes. The onset of unconsciousness was usually preceded by unintelligible speech, a lull in conversation and activity, and loss of sight before hearing and touch. Unconsciousness appeared to occur suddenly.

The objective symptoms varied to some extent with the environmental conditions, especially when using the lower effective concentrations of nitrous oxide. Visual stimuli seemed to create trains of thought and the subject was more noisy and obstreperous when the eyes were kept open than when they were closed, as they frequently were, to avoid the distracting influences of laboratory equipment. Moreover when experiments involving concentration of effort were made during the

inhalation of 50 per cent nitrous oxide, talkativeness and laughter were generally absent.

Short inhalations of pure nitrous oxide were also given. In these experiments, which were the earliest made, the pure gas was administered from an ordinary Cattlin bag with attached face-piece. The actual quantity of gas inhaled was consequently not measured but the number of inspirations and the approximate time taken were noted. Objective symptoms were not seen until three deep inspirations lasting twenty seconds were inhaled. With four inspirations, which took from twenty-five to forty seconds according to the depth of the inspirations, symptoms became marked. Laughter and loquacity, with movements of the head and purposeless gesticulations of the hands increasing to waving about of the arms and general restlessness followed. The face was usually flushed and the breathing somewhat deeper than normal. After five inspirations lasting forty to fifty-five seconds, the subject became very voluble and frequently excitable and aggressive. There seemed to be difficulty in articulation and duskiness to decided cyanosis was present.

Inhalations lasting longer than fifty-five seconds were chiefly characterized in the late stages by marked incoördination and consequent inability to write words or phrases, incoherent remarks and uncertain speech, total disinclination for bodily or mental effort, passing into complete unconsciousness with cyanosis and sterterous breathing.

The approximate times of onset of the various symptoms are shown in table 1.

Reference may also be made to the general effects observed in experiments where the concentration of nitrous oxide was changed without intermission of the inhalation. The experiments included the successive inhalation of 70–50–30 per cent; 60–40–20 per cent; 50–30–10 per cent; and of 10–30–50 per cent nitrous oxide. In the first mentioned experiment 70 per cent nitrous oxide was given with the production of the usual symptoms until unconsciousness supervened. The concentration was then changed to 50 per cent. Gradually recovery occurred, the

subject expressing himself later, as having experienced the curious sensation of a reawakening from death to life. Two minutes after the change, uncontrollable laughter set in and was replaced two minutes later by noisy talkativeness. At this time there appeared to be difficulty in visual accommodation but he was able to write the Lord's Prayer in a just legible manner. No further improvement occurred. The inhalation of the 50 per cent nitrous oxide was continued for nine minutes fifty eight seconds and was then replaced by 30 per cent nitrous oxide. Some nausea was complained of immediately after the change. There was no further laughter or noisy talk. Greater

TABLE 1

	CONCENTRATION OF NITROUS OXIDE				
	100 per cent	75 per cent	70 per cent	60 per cent	50 per cent
Commencing intoxication	7″–10″	30″	35″	45″	60″
Marked intoxication	20″	70″	90″	120″	150″
Talkativeness	30″	90″	90″–120″	150″	180″
Noisy talkativeness	40″	2′	3′ –3½′	4′	5′–6′
Aggressive and emotional	55″	3′	3½′–4′	4½′	6′–7′
Incoördination and unintelligible speech	60″	3½′	4′ –5′	5½′–6′	7′–8′*
Unconsciousness	60″–65″	4′–4½′	5′ –6′		

* Incoördination only.

power of concentrating attention on the experiments developed, writing became normal and the Lord's Prayer was written without hesitation. Memory for details however was found to be impaired. The inhalation of 30 per cent nitrous oxide was continued sixteen minutes and under its influence the subject expressed himself as feeling fairly normal.

In the 60–40–20 per cent series, evidence of lack of attention and power of concentration occurred three minutes after the commencement of the 60 per cent nitrous oxide and in six minutes the responses became markedly accentuated and even somewhat violent. At six and a half minutes, the inhalation was changed to 40 per cent Evidence of increasing consciousness rapidly occurred. Two and a half minutes later, writing was in parts

still illegible and there was some difficulty in visual accommodation. The inhalation was continued eight minutes and then changed to 20 per cent. Slight nausea was experienced at first but return to apparent normality quickly followed. Towards the end of the inhalation, which was continued thirteen minutes, memory was found to be only slightly impaired. The series 50–30–10 per cent showed similar, but less marked, results. The inhalation of 50 per cent nitrous oxide for seven minutes produced the usual effects of difficulty in concentration and incoördination, but writing, although bad, remained legible. On changing to 30 per cent nitrous oxide writing became practically normal, memory improved but remained somewhat defective. The substitution of 10 per cent nitrous oxide was followed by apparent normality.

When the series 10–30–50 per cent nitrous oxide was inhaled, no objective symptoms occurred during the thirteen minutes the smallest concentration was administered but its replacement by 30 per cent nitrous oxide, which was inhaled for eleven minutes, caused lack of effort and concentration. The substitution of 50 per cent nitrous oxide produced the usual volubility and inability to hold the head up.

It would thus seem that definite concentrations of nitrous oxide eventually produce fairly constant physiological effects whether inhaled from the first or after the administration of smaller or larger concentrations. Slight variations according to the form of induction naturally occur. Thus the subject intimated a distinct increase in paresthesia on passing from 10 to 30 per cent, and again on passing from 30 to 50 per cent nitrous oxide; but although he complained of paresthesia during the action of 50 per cent nitrous oxide in the 50–30–10 per cent series, no intimation of its being felt was made when the concentration was reduced to 30 and 10 per cent respectively.

EXPERIMENTS ON REACTION TIMES

These included simple reaction times to a light stimulus, and reaction times to a choice of light stimuli.

TABLE 2

COMPOSITION OF THE INHALATION	TIMES AFTER COMMENCEMENT OF THE INHALATION	INDIVIDUAL REACTION TIMES					AVERAGE REACTION TIMES
Normal air		0.24″	0.26″	0.22″	0.22″	0.20″	
		0.24″	0.24″	0.24″	0.26″	0.26″	
		0.24″	0.24″	0.24″	0.24″	0.26″	0.24″
		0.26″	0.26″	0.22″	0.24″	0.26″	
		0.24″	0.22″	0.24″			
70 per cent N_2O + 30 per cent O	60″–120″	0.28″	0.26″	0.30″	0.30″	0.28″	
		0.32″	0.36″	0.36″	0.38″	0.40″	0.34″
		0.30″	0.58″	0.30″			
	120″–180″	0.36″	0.34″	0.44″	0.32″	0.42″	
		0.38″	0.44″	0.84″	0.42″	0.84″	0.48″
Inhalation changed to 50 per cent N_2O + 30 per cent O + 20 per cent N	5′30″	Unconsciousness					
	7″–8′	0.30″	0.30″	0.28″	0.36″	0.40″	
		0.42″	0.38″	0.40″	0.38″	0.84″	0.38″
		0.30″	0.30″	0.40″	0.30″		
	8′–9′	0.34″	0.30″	0.34″	0.32″	0.38″	
		0.32″	0.36″	0.36″	0.40″	0.26″	0.34″
				0.32″			
	23′30″–24′30″	0.32″	0.30″	0.32″	0.38″	0.26″	
		0.36″	0.30″	0.32″			0.32″
	24′30″–25′30″	0.28″	0.30″	0.36″	0.28″	0.34″	
		0.32″	0.30″	0.26″	0.34″	0.32″	0.31″
Inhalation over; air valve turned on	35′30″						
	37′–39′	0.24″	0.26″	0.26″	0.24″	0.26″	
		0.24″	0.28″	0.30″	0.30″	0.24″	
		0.32″	0.30″	0.30″	0.26″	0.28″	0.27″
		0.28″	0.32″	0.26″	0.24″	0.26″	
		0.30″	0.30″				
	44′–46′	0.26″	0.26″	0.26″	0.26″	0.24″	
		0.26″	0.24″	0.40″	0.24″	0.26″	0.26″
		0.24″	0.26″	0.24″	0.28″	0.26″	
		0.26″	0.24″	0.26″	0.30″	0.30″	
				0.24″			

Simple reaction times

The apparatus employed consisted of an electric bulb controlled by a mercury key, the time and duration of the flash being registered on a revolving drum. The response was made by a tapping key which registered immediately below the rec-

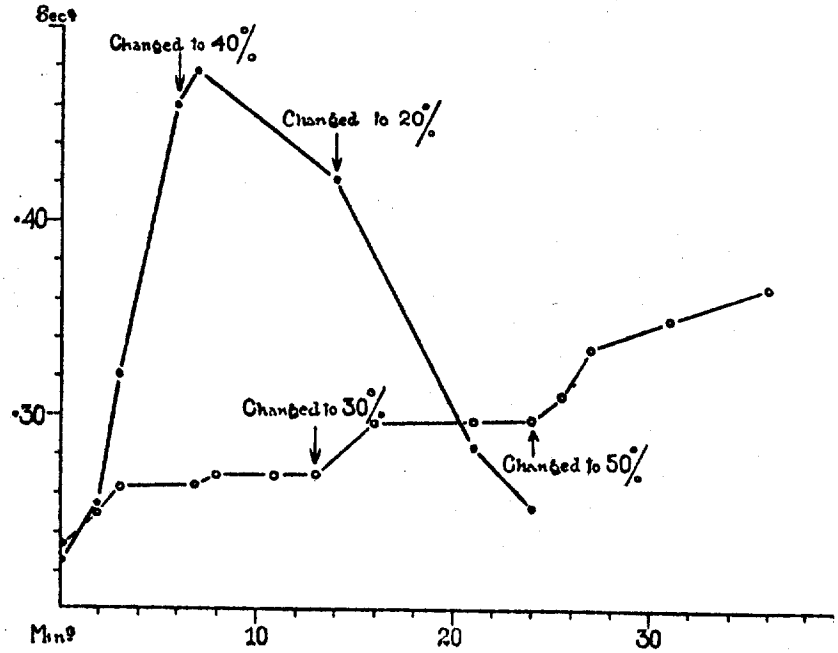

FIG. 1. GRAPH SHOWING EFFECT OF SUCCESSIVE ADMINISTRATIONS OF NITROUS OXIDE ON SIMPLE REACTION TIMES

The arrows indicate the times when the concentrations were changed. Ordinates = reaction times in seconds. Abscissae = time in minutes from commencement of the inhalation.

ord of the light stimulus. Except where the condition of the subject was rapidly changing, a large number of observations were made and after measurement were averaged. In the graphs this average has been plotted at the end of the time periods. The time taken in the travel of the tapping lever from rest to contact which amounted to 0.05 second has been ignored.

As an example of an experiment and of the variations in the reaction times obtained, the observations made during an inhalation of 70 per cent followed by 50 per cent nitrous oxide may be taken. These are given in table 2. The inhalation of 70 per cent nitrous oxide was carried to complete unconsciousness, which resulted after five and one-half minutes, but records were only made during the first three minutes of the inhalation. When unconsciousness occurred, the inhalation was replaced by 50 per cent nitrous oxide and the reaction times were continued. Thirty-five and one-half minutes after the commencement of the inhalation the air valve was turned on and the reaction times taken during recovery. In another experiment of a similar kind the reaction times were carried nearer unconsciousness. The records towards the end became markedly prolonged and sometimes no response was given. The times obtained from one hundred and eighty to two hundred and thirty-one seconds after commencing the inhalation of 75 per cent nitrous oxide were as follows:

0 = no response; + = erroneous reply, i.e., reply without any preceding stimulus.

TIME AFTER COMMENCING THE INHALATION	REACTION TIMES						
180″–231″	0.42″	0.40″	0.36″	0.40″	0.42″	+	0.36″
	0.30″	+	0.64″	+	0.82″	0.38″	0.36″
	0.44″	0.42″	0.42″	0.36″	0.36″	0.78″	0.90″
	0.38″	0.44″	0	1.6″	0	2.0″	0
				3.1″			

No further responses were obtained. Unconsciousness occurred at two hundred and sixty seconds, twenty-nine seconds after the last response.

The results obtained from the inhalation of a 60–40–20 per cent series and of a 10–30–50 per cent series are shown on the graph (fig. 1). The marked rise in the reaction times during the inhalation of 60 per cent nitrous oxide is followed by a fall on changing to 40 per cent and a much more marked fall on changing to 20 per cent. Conversely, during the inhalation of the 10–30–50 per cent series, there is an increase in the reaction

times with each increment of nitrous oxide. Usually each concentration of nitrous oxide if sufficiently long inhaled produced an average reaction time which was fairly constant. This is indicated in the 10–30–50 per cent series but is not seen in the 60–40–20 per cent series as the respective concentrations in this experiment were not administered for a sufficient length of time to produce a constant effect.

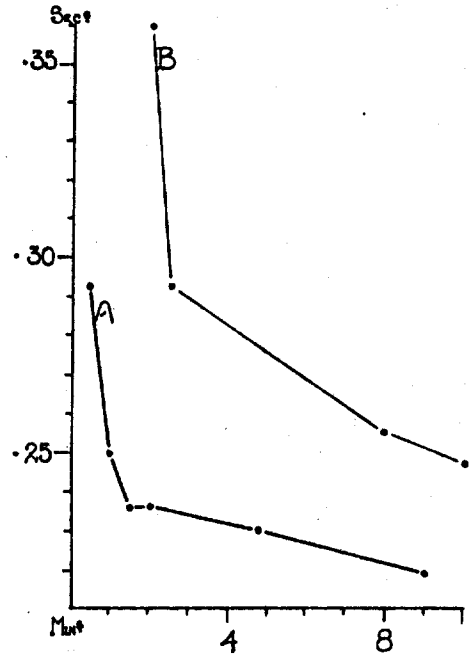

FIG. 2. GRAPH SHOWING SIMPLE REACTION TIMES DURING RECOVERY FROM MILD (*A*), AND SEVERE (*B*) INTOXICATION WITH NITROUS OXIDE

Ordinates = reaction times in seconds. Abscissae = time in minutes after cessation of the administration.

The simple reaction times during recovery from pure nitrous oxide inhalations of ten seconds duration and up to complete unconsciousness are plotted in the graph (fig. 2). Reaction times were commenced as soon as possible after completion of the inhalation and both showed rapid recovery towards normality. The deeper intoxication naturally shows the slower return. But with the degrees of intoxication produced by

inhalations of nitrous oxide up to forty seconds duration, the curve of recovery for simple reaction times was very similar to that from mild intoxication. The reaction time did not in any case return to normal during the time the experiments were continued; a fact probably to be associated with the prolonged feeling of depression after each experiment which was recorded by the subject.

Reaction times with choice

The apparatus was similar to that used for simple reaction time but included two lighting circuits—one with yellow or green light, and the other with red light—arranged in contact and controlled by mercury keys. The response was to a chosen light and was recorded as in the experiments on simple reaction time.

The normal choice reaction time was 0.26 second. The reaction times taken before some of the experiments, as may be seen in the graphs, was often longer than this owing to the subject not having completely recovered from the previous experiment. The reaction time with choice was not materially influenced by the inhalation of concentrations of nitrous oxide up to 20 per cent. The greatest effect observed with an inhalation of 10 per cent nitrous oxide was an increase of 17 per cent.

An example of the results obtained with this form of reaction time is seen in the graph (fig. 3). This illustrates the effect of three inhalations of 30, 40 and 50 per cent nitrous oxide given successively with an interval of one hour between the first and second, and of two hours between the second and third inhalations. It is evident from comparison with the graph of simple reaction times that the reaction involving choice is much more affected by nitrous oxide than the simpler reflex. This is clear from the comparison of the graphs of the inhalation of 50 per cent nitrous oxide. Notwithstanding that the inhalation of 10 and 30 per cent nitrous oxide had preceded it without intermission, the simple reflex time was only increased from the normal of 0.23 second to one of 0.37 second (61 per cent increase), whereas in the case of the choice reflex time with discontinuous

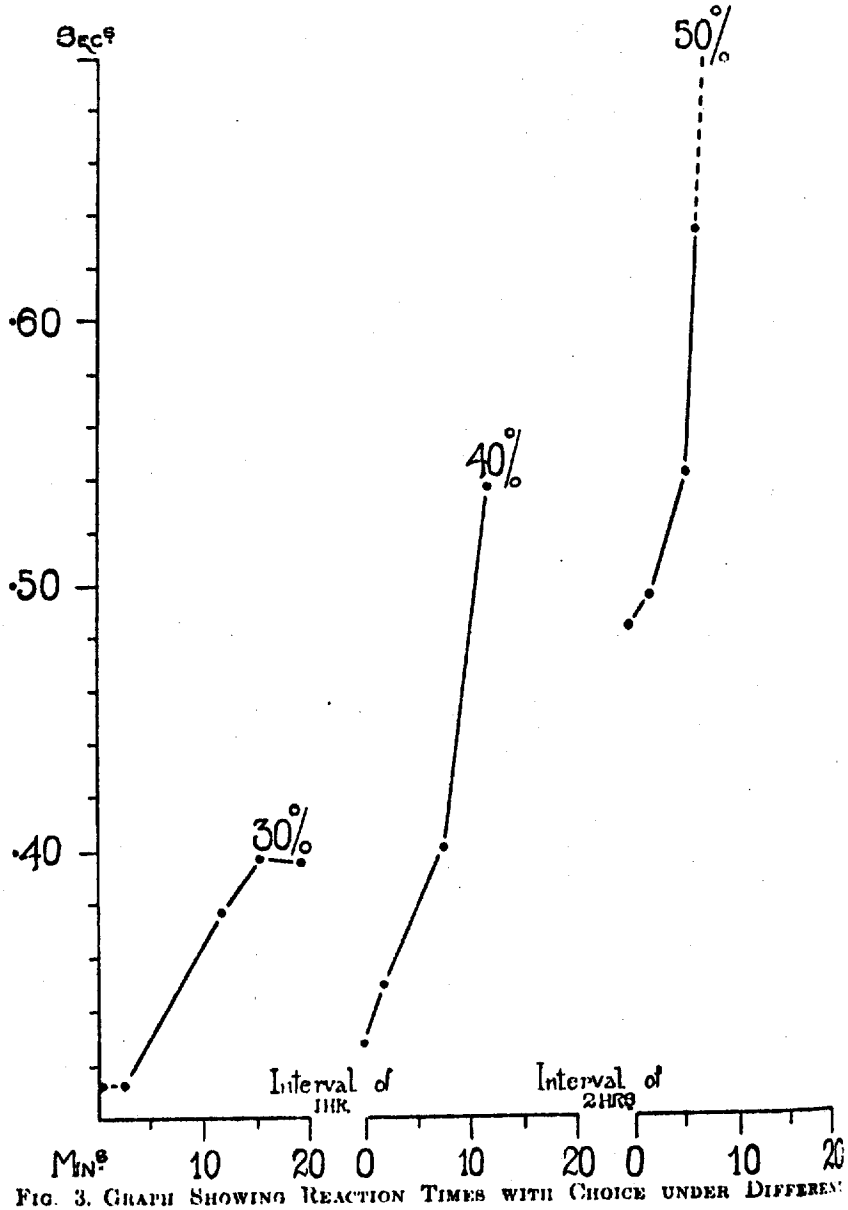

FIG. 3. GRAPH SHOWING REACTION TIMES WITH CHOICE UNDER DIFFERENT CONCENTRATIONS OF NITROUS OXIDE

Ordinates = reaction times in seconds. Abscissae = time in minutes after the commencement of each successive administration.

administration of 30, 40, and 50 per cent nitrous oxide, the choice reaction was increased from a normal of 0.31 second to one of 0.65 second during the 50 per cent inhalation (110 per cent increase). Moreover, choice reflex is more influenced by smaller concentrations of nitrous oxide than the simpler reflex.

The results obtained during recovery from inhalations of pure nitrous oxide of varying duration, are shown in table 3. The mild intoxication was induced by two inhalations of nitrous oxide lasting ten seconds, and the moderate intoxication by four inhalations lasting thirty-five seconds. The last column shows the effect following intoxication to complete unconscious-

TABLE 3

TIME AFTER CESSATION OF THE INHALATION	MILD INTOXICATION	MODERATE INTOXICATION	INTOXICATION TO UNCONSCIOUSNESS
15″–30″	0.34″	0.35″	
30″–60″	0.31″	0.35″	0.39″
60″–120″	0.28″	0.34″	0.39″
240″–420″	0.27″	0.32″	0.30″

TABLE 4

TIME AFTER CESSATION OF THE INHALATION	CHOICE REFLEX	CHOICE REFLEX WITH DURATION	NORMAL CHOICE REFLEX WITH DURATION
30″–60″	0.39″	0.65″	
60″–120″	0.39″	0.53″	0.27″
240″–420″	0.30″	0.48″	

ness. The normal reaction time for choice on this occasion was 0.26 second. As will be seen from a comparison with the graph (fig. 2), showing the effect on simple reaction time, reaction time with choice is more influenced by, and the return towards normal is less rapid from, similar degrees of intoxication. Besides an increase in the time taken, mistakes were occasionally made in the response. In the calculations these have been ignored.

The introduction of conscious effort appears to increase very materially the reaction time. Thus, in one experiment where the subject was asked to respond to the duration as well as the kind of light, the reflex times were nearly doubled. This is

seen in table 4 which shows the results obtained after intoxication to complete unconsciousness.

In this experiment, the duration of the light stimulus was, under normal conditions, slightly underestimated for all times. Under intoxication it was slightly overestimated for signals of shorter duration than one second, and was underestimated to a greater degree than normally, for signals of longer duration than one second.

EXPERIMENTS INVOLVING MUSCULAR MOVEMENTS

These experiments although varying in psychological significance are conveniently associated together; and a brief account of observations on equilibration may precede the description of experiments in which registration of movements was made. This was determined in many experiments immediately after cessation of the inhalation by asking the subject to walk along a straight line. Slight ataxia was noted after the inhalation of 20 per cent nitrous oxide but it was not until after the inhalation of 30 per cent that inability to walk straight occurred. After 40 per cent nitrous oxide the gait was unsteady but to some extent could still be controlled. This appeared to be impossible after the inhalation of 50 per cent and after 65 per cent nitrous oxide there was inability to walk without support.

The experiments in which muscular efforts were registered included ergographic, tapping, pricking, writing and typewriting.

Ergographic records

These experiments were undertaken primarily to study the effect of unskilled work on fatigue. The method had eventually to be discarded owing to the facilitation which was acquired and which made accurate comparisons difficult. The weight raised in the earlier experiments was 1 kgm.; later it was increased to 3 and 5 kgm. Under 10 per cent nitrous oxide and 18 per cent oxygen and a load of 3 kgm., the total work accomplished was somewhat less than under normal conditions. There were slight spurts of increased activity from time to time and

fatigue was delayed. The terminal contractions, however, became ineffective long before cessation occurred. Normally, the contractions ceased while moderately efficient owing to pain as well as exhaustion. Under 30 per cent nitrous oxide and 14 per cent oxygen the total work accomplished was approximately the same as normal mainly owing to delay in fatigue. Occasional spurts of slight increased activity occurred with this strength also. The results with this load are shown in table 5. Using a load of 5 kgm. the effect obtained with 10 per cent nitrous oxide was again a marked delay in the onset of fatigue. The initial contractions were smaller but the total work performed was a little greater than normal. With 40 per cent nitrous oxide there was also a delay in fatigue and a noteworthy

TABLE 5

INHALATION	TIME OF EXHAUSTION	NUMBER OF CONTRACTIONS	WORK DONE IN KILOGRAM METERS
Air	110″	169	6.24
10 per cent N_2O	151″	172	6.00
30 per cent N_2O	160″	210	6.27

TABLE 6

INHALATION	TIME OF EXHAUSTION	NUMBER OF CONTRACTIONS	WORK DONE IN KILOGRAM METERS
Normal air	47″	75	5.20
10 per cent N_2O	77″	142	5.91
40 per cent N_2O	64″	120	8.26

increase in the work performed. This was the best effect obtained under nitrous oxide intoxication. The results are given in table 6. In this series of experiments, 30 per cent nitrous oxide was administered between the 10 and 40 per cent inhalations. This has been omitted in the table because it produced less work than normal, a result which appeared to be due not to fatigue but to disinclination on the part of the subject to continue the work. Indeed, he remarked on this occasion that he did not see the necessity of working.

The number of ergographic experiments made, was too small to draw very satisfactory conclusions from, but they seemed to

show that the work performed before facility had been acquired was more uniform under intoxication than under normal conditions. The initial work was usually less but the delayed fatigue generally produced a greater total amount of work. There is

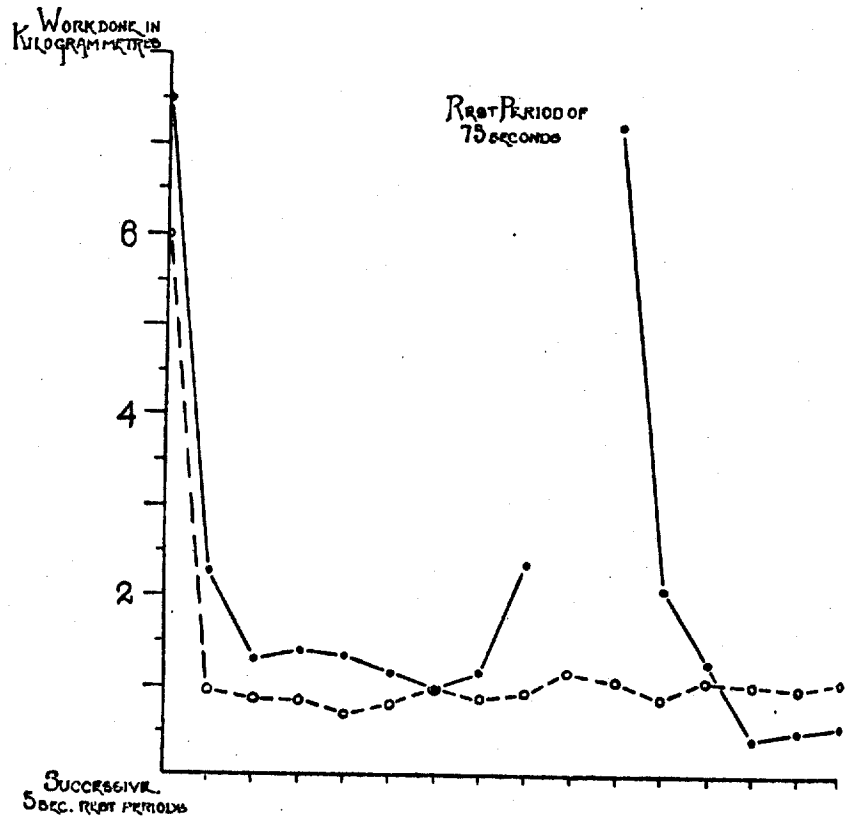

FIG. 4. GRAPH SHOWING THE EFFECT OF 30 PER CENT NITROUS OXIDE ON MUSCULAR WORK AFTER FIVE SECOND PERIODS OF REST

Ordinates = work done in kilogram meters. Each division of the abscissa represents rest periods of five seconds.

however, under intoxication, less reserve of energy and less ability for intensive work than under normal conditions. The uniformity of work under moderate intoxication (30 per cent nitrous oxide) is shown in the graph (fig. 4). This figure illustrates by the broken line, the initial work performed, and

that obtained after successive periods of five-second rests under intoxication. The continuous line represents the work done in a normal state under similar conditions. In the normal experiment at the end of the seventh five-second period of rest, a special effort was made and resulted in twice the amount of work being accomplished. After this a seventy-five seconds rest was given. The subsequent work period shows a great increase in the amount of work performed but the exhaustion induced by the effort caused the work following each five-second period to fall below that during intoxication. In the experiment under intoxication a rest period of sixty seconds was given immediately after the observations recorded in the graph. The work accomplished after this period of rest was only 1.33 kgm. meters as compared with 0.86 kgm. meter after the preceding five seconds rest. Moreover, after this slight increased effort the work performed after each succeeding five second period gradually diminished. In most experiments there has been relatively little difference in the work done after brief periods of rest whether in a normal or intoxicated state.

Under moderate intoxication there seemed to be an endeavor to economize energy. This was seen not only in ergographic experiments but also in experiments on reaction times. The force used in tapping the recording key was generally less than under normal conditions and in many instances was even insufficient to ensure contact. On the other hand, under intoxication, the facility obtained by practice seemed to be less rapid than under normal conditions. The average work performed in a normal state before exhaustion was reached was in the early ergographic records, 8 kgm. meters. In the last record made it was 15 kgm. meters. No similar increase was observed under intoxication.

It would thus seem from these experiments that during moderately severe intoxication, unskilled work can be performed efficiently. The work is less strenuous than in a normal state, but the work is maintained at a more uniform level and there is delay in the onset of fatigue.

The ergographic experiments during the inhalation of pure

nitrous oxide were also few in number and the results were similar. After the inhalation of a single breath of the gas, the work accomplished was sometimes greater, sometimes less than normal and there was evidence of diminished fatigue. After five breaths of pure nitrous oxide the contractions became somewhat irregular but continued of approximately normal height for twenty to twenty-five seconds and then gradually fell and ceased twenty-five seconds later. A period of about a minute broken by a few spasmodic contractions followed. On one of the three occasions on which this quantity was inhaled the sustained cessation was due to forgetfulness of the experiment since the subject, when told to continue, at once worked efficiently.

Tapping movements

Tapping experiments guage chiefly the effect on rapidity of movement. Some early experiments of a preliminary nature were carried out by asking the subject to tap once a second on the knee. These however were employed mainly to determine roughly on a simple and easy movement, the degree of coördination and the power of concentration. With the inhalation of 50 per cent nitrous oxide and 50 per cent oxygen this simple movement became more violent after four minutes and the subject commenced to count the taps in tens, making ten rapid and ten slow taps alternately until six minutes after the beginning of the inhalation when he lost count. The tapping movements ceased and he commenced to talk loudly and to wave his arms about violently. When told to continue the tapping he said he had not forgotten. A repetition of the experiment produced similar results.

With an inhalation of 70 per cent nitrous oxide and 30 per cent oxygen the tapping movements began to increase in one minute after commencing the inhalation, and three minutes later they became aggressive and violent. In another half minute, they became incoördinate falling wide of the knee. Unconsciousness occurred six minutes after the commencement of the inhalation. Similar effects, which however appeared somewhat earlier, were

produced with inhalations of 75 per cent nitrous oxide and 25 per cent oxygen.

The experiments on the rapidity of tapping movements were made with a square metal disc of 11.2 mm. diameter, fixed in a wooden board, and a metal pencil of 2.2 mm. diameter which were included in an electric circuit. Contact was registered by a signal on a revolving drum and the taps were made by free arm movement. The rapidity of tapping was not materially influenced by moderate intoxication so that the effect of severe intoxication, alone need be described. This was induced by the inhalation of 70 per cent nitrous oxide, and 30 per cent oxygen. The results are shown in table 7. Incoördination commenced after two minutes and, as the intoxication became very deep, the taps fell wide of the plate and in consequence

TABLE 7

TIME FROM COMMENCEMENT OF THE INHALATION	NUMBER OF TAPS IN SUCCESSIVE 5-SECOND PERIODS						AVERAGE
Normal							34
30″–60″	35	32	31	31	31	32	32
60″–90″	30	30	29	30	30	28	29.5
90″–120″	30	29	28	28	28	27	28.3
120″–145″	25	24	23	22	21		23
280″–300″	23	23	10	1			

were not always recorded. There was however marked slowing. The two 23's successively recorded in the last line of the table were obtained under a request to work hard and they seemed to indicate that even in deep intoxication some concentration on such a simple movement was possible.

Pricking experiments

These were employed to determine the effect on the accuracy of simple muscular movements. The apparatus consisted of a plate bored with circular holes of different sizes and a cylindrical metal pin of smaller size fixed into a handle which were introduced into an electric circuit containing also a recording signal arranged to register on a revolving drum. A record was made whenever the metal pin, during the pricking of the hole, failed

to make perfect clearance. The diameters of the tap holes used were 2.74 mm. and 3.55 mm., the diameter of the pin was 1.06 mm. The pricks were made usually with free as

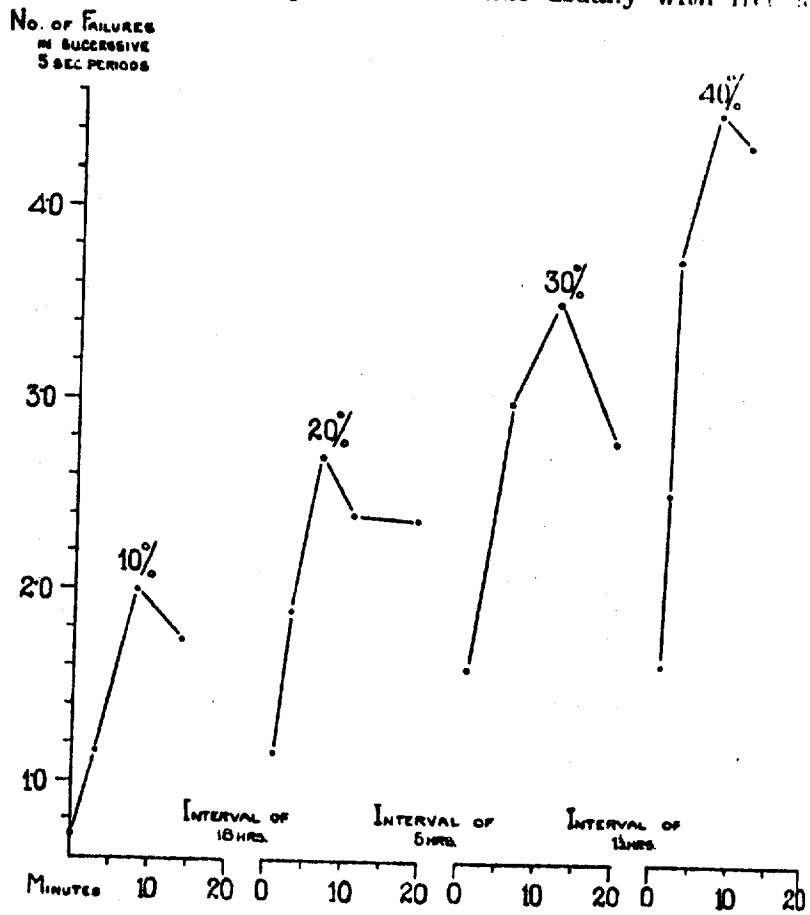

Fig. 5. Graph Showing Effect of Different Concentrations of Nitrous Oxide on Accuracy of Muscular Movement as Indicated by Pricking Experiments

Ordinates = number of failures to make perfect clearance in successive five-second periods. Abscissae = time in minutes after commencement of each successive administration.

movement to the sound of a metronome arranged to beat every second. With the 2.74 mm. tap hole, the mistakes averaged,

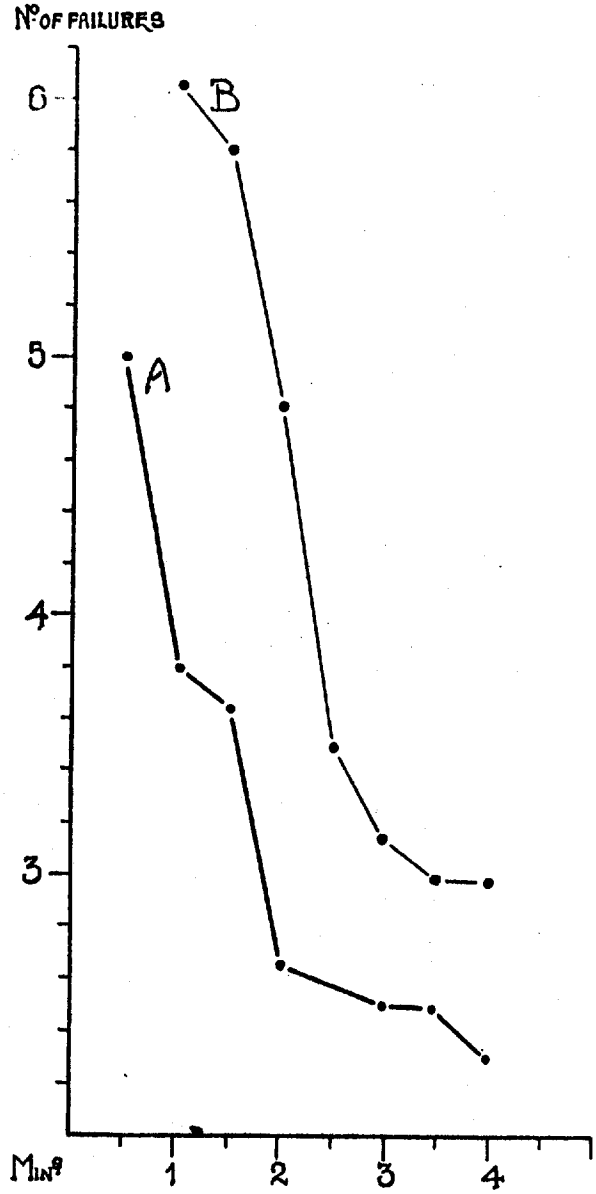

FIG. 6. GRAPH SHOWING IMPROVEMENT IN ACCURACY OF MUSCULAR MOVEMENT DURING RECOVERY FROM MILD (*A*) AND MODERATE (*B*) INTOXICATION WITH NITROUS OXIDE

Ordinates = number of failures to make perfect clearance in successive five-second periods. Abscissæ = time in minutes after cessation of the inhalation.

under normal conditions, 0.7 in a five-second period. The mistakes which occurred during the action of 10, 20, 30 and 40 per cent nitrous oxide are shown in the graph (fig. 5). The figure shows an increased number of failures with each increase in concentration of nitrous oxide, and it further shows in each instance a slight improvement after the acme of error has been reached. This improvement has been too constant to be accidental and it suggests that even in fairly advanced intoxication some degree of education is possible. Sometimes the hole was completely missed. Under the influence of 10 per cent nitrous oxide this occurred once in 14.5 times; under 20 per cent once in 11 times; under 30 per cent once in 7.5 times and under 40 per cent once in 1.3 times. These complete failures in percentages being 7, 9, 13, and 77 respectively.

Accuracy of movement is thus more readily influenced by a condition of intoxication than is simple unskilled movement. Under 50 per cent nitrous oxide a fair amount of simple mechanical work is still possible but even with 40 per cent nitrous oxide all accuracy of fine movement may be said to have been lost.

Comparable results were obtained during recovery from inhalations of pure nitrous oxide and are shown in the graph (fig. 6). The earliest records were very irregular and it was obvious that the metronome was being ignored.

Writing

In the act of writing, want of coördination commenced with the effect of 50 per cent nitrous oxide. Concentrations less than this had no distinct action. The inhalation of 60 per cent nitrous oxide caused marked incoördination and with the inhalation of 65 per cent the writing soon became illegible and eventually terminated in rapid scrawls. In the successive 70–50–30 per cent series of nitrous oxide inhalations the writing became legible but remained more scrawly than normal, three minutes after the change from 70 to 50 per cent; and in the change from 60 to 40 per cent nitrous oxide in the 60–40–20 per cent series the writing still showed badly formed letters three minutes after the change. With the changes to the lower concentrations

the writing became normal. As long as remembrance of events was retained, legible writing seemed possible. Conversely, as long as the subject was able to write at all legibly, he was able to answer questions given him. This was investigated by keeping him in this state by administering 70 per cent nitrous oxide with occasional breaths of air.

Typewriting

Typewriting involves complex psychological processes but in the present connection the experiments are chiefly of interest in relation to the associated muscular coördination. The

TABLE 8

COMPOSITION OF THE INHALATION	TIME AFTER THE COMMENCEMENT OF THE INHALATION	TIME TAKEN TO TYPE 4 SENTENCES	NUMBER OF MISTAKES MADE
Air		96″	1
30 per cent N_2O + 30 per cent O	90″–201″	100″	0
	3′55″–5′48″	102″	4
	5′55″–7′44″	100″	6
Changed to 50 per cent N_2O + 26 per cent O at	8′		
	9′33″–11′42″	121″	7
	12′18″–14′47″	138″	15
	15′28″–18′	143″	23

subject was not a proficient typist. He had employed a machine for personal purposes many years ago but had discontinued its use. Typewriting was consequently an unusual occupation and required more alertness than ordinary writing. Nevertheless, distinctly defective coördination commenced as in ordinary writing with the inhalation of 50 per cent nitrous oxide. Percentages lower than this showed variations from the normal chiefly in the number of mistakes made, to a less extent in the time taken, and these points may be conveniently referred to here. A short sentence requiring 71 movements was given and was practised to obtain efficiency. Table 8 shows the effect of 30 per cent followed by 50 per cent nitrous oxide. The

greater time shown in the second as compared with the third column is due to the time taken in setting the machine for separate lines. With the inhalation of concentrations of nitrous oxide up to 30 per cent, the time taken did not vary materially from the normal. Concentrations below this affected only errors, which were decidedly increased even during the action of 10 per cent. The inhalation of this strength increased the errors from a normal of two for five sentences to one of four, five and nine during successive four minutes periods of inhalation, and fell again to one, during the second four-minute period of recovery.

EFFECT OF MEMORY

This was tested by graded experiments including the writing of the Lord's Prayer, the answering of a number of general questions, the remembrance of disconnected words, of words associated in pairs, in series of five and nine, the spelling of words, and the estimation of time periods. As the experiments are chiefly of psychological interest, a few of the more important results need only be mentioned.

Remembrance of the Lord's Prayer was complete under 50 per cent nitrous oxide. Even when this concentration followed the inhalation of 70 per cent to unconsciousness, the Prayer was given correctly with the omission of "Lead us not into temptation." With the inhalation of 60 per cent complete failure of memory of the Lord's Prayer occurred within three minutes of the beginning of the inhalation but a sentence of it was written to dictation four minutes later. Incoördination with inability to hold the pen followed. In every experiment, the time taken to write the Lord's Prayer under intoxication and during recovery from intoxication was longer than under normal conditions.

A series of ten general questions and their answers were committed to memory before the beginning of the experiments and were used at infrequent intervals. The remembrance of these formed a more difficult test than the remembrance of the Lord's Prayer. The concentration of nitrous oxide at which memory failed for them was approximately 50 per cent. During the

inhalation of this strength following the administration of 70 per cent to unconsciousness, the questions were remembered with difficulty, the answers being arranged in an unusual order in one experiment and being incomplete in a second experiment. While under the influence of 40 per cent nitrous oxide following the inhalation of 60 per cent, the questions were remembered correctly and in proper order although the time taken to write the answers was longer than normal. With smaller concentrations the questions and answers were readily remembered but occasionally they did not appear to come up into consciousness at once, and after the inhalation of 30 per cent nitrous oxide for seventeen minutes in one experiment, two of the ten questions could not be recalled.

In the tests made during recovery from inhalations of pure nitrous oxide, it was found that the first answer written correctly and in the time normally taken was forty, sixty-four, and one hundred and sixty seconds respectively for moderate, severe, and very severe intoxication.

Varied experiments with word series also brought out the fact that the more difficult the test, the smaller the concentration of nitrous oxide required to affect it; and similarly, while the spelling of most words was given correctly under 60 per cent nitrous oxide, the spelling of "Llandudno" occasionally failed at 40 per cent.

Time estimations

If methods of dividing the time such as counting are excluded, time estimations probably form one of the most difficult of memory tests. These experiments included the estimation of five, ten, and fifteen seconds during the inhalation of different concentrations of nitrous oxide and during recovery from inhalations. In a few instances under severe intoxication counting was permitted, otherwise no accessory aid to the time estimation was allowed, and according to the subject's admission was not employed. Experiments were frequently made in the intervals of other observations but in some experiments the time estimations were recorded on a revolving drum and afterwards measured.

An example is graphed in figure 7. This shows the results of estimating ten-second periods under the influence of 20, 30, and 40 per cent nitrous oxide taken during the same day. An interval of three hours occurred between the inhalations of 20

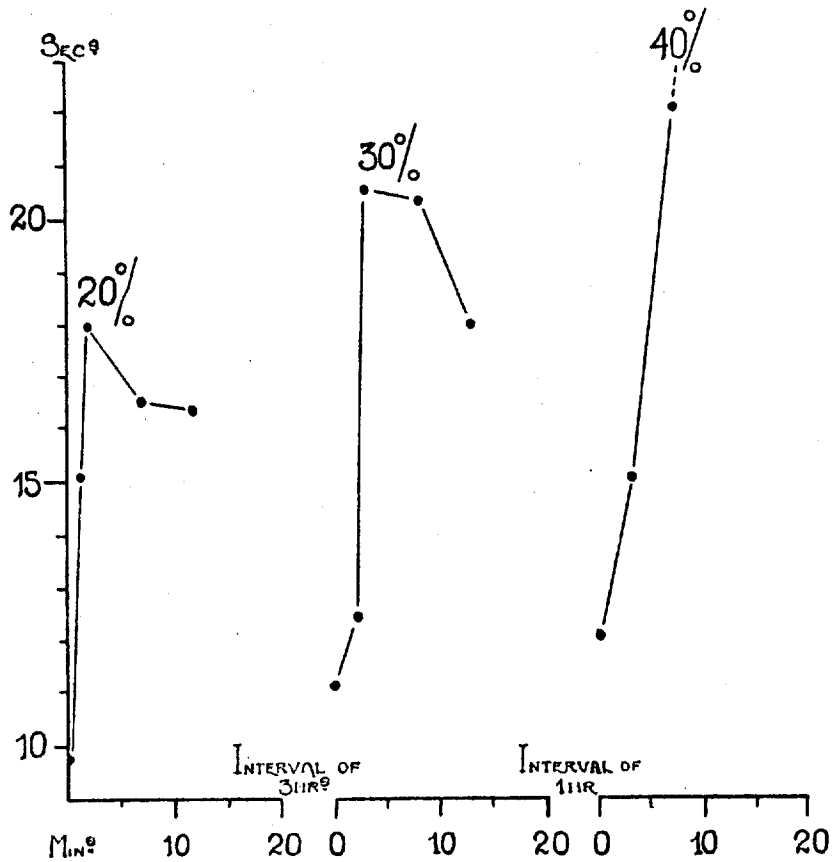

Fig. 7. Graph Showing Effect of Different Concentrations of Nitrous Oxide on the Estimation of Ten-Second Periods

Ordinates = estimated time in seconds. Abscissæ = time in minutes after the commencement of each successive inhalation.

and 30 per cent and an interval of one hour between the inhalations of 30 and 40 per cent nitrous oxide. The average normal estimation of ten seconds was 9.8 seconds. This increased to 15.1 seconds after one minute inhalation of 20 per cent nitrous

oxide, and to 18 seconds after three minutes inhalation. Later it fell and then remained fairly constant. The subsequent inhalation of 30 and 40 per cent nitrous oxide caused a slower rise in error but eventually the error was greater, the estimation after eight minutes inhalation being 20.6 seconds under 30 per cent and 22.1 seconds under 40 per cent nitrous oxide. Considerable variations were shown, and with the more marked intoxications the irregularities suggested pure guessing. The extremes of the estimation of the ten-second period after intoxication had been established were, for 20 per cent nitrous oxide 14.1 and 20.1 seconds; for 30 per cent nitrous oxide 14.5 and 25.5 seconds; for 40 per cent nitrous oxide 18.6 and 26.1 seconds.

TABLE 9

TIME AFTER CESSATION OF THE INHALATION OF PURE NITROUS OXIDE	AVERAGE ESTIMATION OF 5 SECONDS		AVERAGE ESTIMATION OF 15 SECONDS	
	Severe intoxication	Intoxication to unconsciousness	Severe intoxication	Intoxication to unconsciousness
10″–30″	10.9″			
30″–60″	8.7″	10.9″		
60″–90″	8.8″	10.8″	40.8″	
90″–120″	7.8″	10.1″	18.6″	43.0″
120″–180″	7.0″	10.0″	17.4″	37.0″
240″–480″		9.8″		27.5″

In the experiment with 40 per cent nitrous oxide the character of the estimations was changed at the end of eight minutes and counting was allowed. Fairly accurate estimation of ten-second periods were then obtained, the result at first being 10.3 seconds and, towards the end of the experiment when the inhalation had continued for twenty-one minutes, the estimated period became less than normal, being 7.9 seconds. With smaller concentrations than 40 per cent, the estimation of ten seconds by counting was approximately accurate.

As might be expected, the longer the time period given for estimation, the more difficult was the problem and the more erratic the replies. This may be illustrated by observations made with five and fifteen second periods during recovery from moderately severe and deep intoxications. In the one set of

experiments pure nitrous oxide was inhaled for 55 seconds, in the other experiments to complete unconsciousness. The results of the time estimations during recovery are given in table 9. Many of these times represent single observations but the tendency of the intoxication to prolong the time estimation especially with the longer interval is distinctly seen. An analysis of the observed times also shows the erratic nature of the response when long periods were employed. Thus while the different individual estimations for five-second periods were fairly uniform, the extremes obtained for fifteen-second intervals, during the two- to three-minute period of recovery from severe intoxication, were 7 and 31.3 seconds.

In conclusion some observations on tactile sensibility and the perception of pain may be mentioned. These were found to be less affected than was expected. Pricking and pinching were felt as pain almost to the state of unconsciousness but, as far as could be determined, with less acuity than normal. Forgetfulness of pain was unquestionably present. Light touch was lost under the influence of 50 per cent nitrous oxide.

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A METHOD OF GAS CHROMATOGRAPHY FOR QUANTITATIVE ANALYSIS OF BLOOD-GASES

BY

D. DENISON DAVIES

SUMMARY

A gas chromatographic system for the quantitative analysis of blood-gases has been constructed. The system incorporates a simplified "equilibration" blood-gas extraction and injection unit, and adsorption columns linked in parallel. Eighty μl. of blood is required for each analysis. The time required to carry out duplicate analyses is 8 minutes. Blood oxygen, carbon dioxide and nitrous oxide contents are determined using peak height measurements. Calibration for carbon dioxide is carried out by using carbonate solution, for oxygen by using room air, and for nitrous oxide by using cylinder nitrous oxide. Reproducibility studies were carried out by analyzing one blood sample ten times: coefficients of variation for oxygen, carbon dioxide and nitrous oxide were, in each case, approximately 0.5 per cent. Comparisons of oxygen and carbon dioxide contents of twelve blood samples were simultaneously determined by the gas chromatographic method and by the Van Slyke manometric method. There was close agreement between the two methods; the coefficients of correlation of the respective estimations were +0.999 in the case of oxygen and +0.995 in the case of carbon dioxide.

Analysis of blood-gases by a gas chromatographic technique involves four main stages: the extraction of the gases from the blood using Van Slyke reagent and their subsequent injection on to the chromatographic columns; separation in the columns of the gas mixture into its individual components utilizing selective adsorption on solid adsorption media; detection of the individual gases by means of a katharometer utilizing thermal conductivity changes; and finally recording of the signals produced by the katharometer (fig. 1).

The extraction of gases from the blood and their injection on to the columns in a reproducible manner has presented the most difficult technical problem encountered in this analytical technique. A "vacuum extraction" method has been described by Ramsey (1959), Hamilton (1959), Lucas and Ayres (1961), Edwards and Farhi (1961), Muysers, Siebolt and Worth (1961), and Farhi, Edwards and Homma (1963). This involves the use of a modified Van Slyke apparatus and therefore suffers from many of the disadvantages and problems inherent in this technique of blood-gas analysis. A "washout" method in which an attempt is made to flush the total volume of the liberated blood-gases into the columns by bubbling carrier gas through the blood-reagent mixture has been described by Wilson and associates (1961), Chambliss and Nouse (1962), Galla and Ottenstein (1962), Hamilton (1962), Johns and Thompson (1963), Hill (1966), Lenfant and Aucutt (1966). This method produces sharp and reproducible peaks and has much to commend it. However, foaming of the reagent is a troublesome problem, the type of apparatus needed is difficult to clean and the replacement of expendable parts is not easy. An "equilibration" method in which the liberated blood-gases are equilibrated between a fixed volume of reagent and a fixed volume of carrier gas, the gaseous fraction alone being then injected on to the columns, has been described by Hamilton (1962). The method gave very sharp peaks but the apparatus used was fairly complex and difficulty was experienced in obtaining a reproducible sample size when gas samples were

D. DENISON DAVIES, F.F.A.R.C.S., Research Department of Anaesthetics, Royal College of Surgeons of England, Lincoln's Inn Fields, London W.C.2.

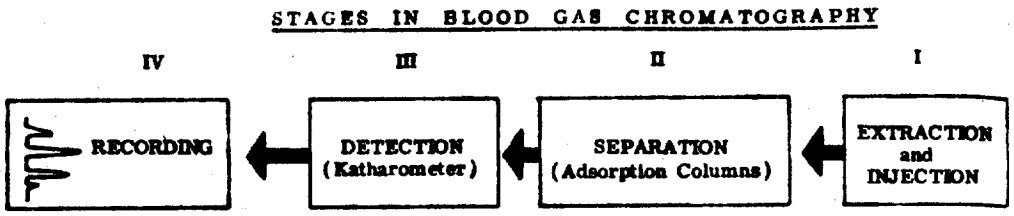

FIG. 1
The four stages of gas chromatography in the quantitative analysis of blood-gases.

introduced for the purposes of calibration. Another type of "equilibration" technique in which only a fixed fraction of the gaseous fraction is injected on to the columns has been described by Ortega and Tammeling (1968).

Separation of the gas mixture utilizing selective adsorption on solid adsorption media is complicated by the fact that no one single adsorbent can be used to separate a mixture of oxygen, nitrogen, carbon dioxide and nitrous oxide. In practice two different solid adsorbents packed in separate columns are used. The two columns can be linked in series (Hamilton and Kory, 1960; Wilson et al., 1961; Hamilton, 1962; Galla and Ottenstein, 1962; Bowes, 1964; Ortega and Tammeling, 1968), or they can be linked in parallel (Hill, 1960, 1966; Johns and Thompson, 1963; Theye, 1964; Lenfant and Aucutt, 1966; Fothergill, 1968). Both types of arrangement have their advantages and disadvantages. With columns in series it is necessary to alter the attenuation of the katharometer, and usually its polarity also, during the course of each individual analysis. The use of columns in parallel is, therefore, preferable if automatic operation is desired. The construction of a suitable pair of parallel columns is, however, more difficult and more time-consuming than in the case of columns in series.

The detection of the components of the gas mixture by means of a katharometer presents no special difficulties. These instruments usually give a strictly linear response over the range of concentrations used but, because of the limit to the sensitivity of the instrument, it is necessary to use a blood sample size of at least 50 μl.

The signals produced by the katharometer can be displayed on a chart recorder, and peak heights, measured manually, can be used for quantification of the individual gases. Peak height may also be determined by using a digital voltmeter with a peak-holding device (Fothergill, 1968) but this method demands a considerable degree of baseline stability and its accuracy is dependent upon the number of decimal places used in the measurement. Peak areas can be determined but where the peaks are sharp and narrowly based there is, so far as reproducibility and accuracy are concerned, probably little to be gained by the measurement of this parameter. The use, however, of an automatic integrater will give the advantage of a digital readout but, again, baseline stability is necessary and the number of decimal places used for measurement must be adequate.

In an attempt to overcome the problems encountered by other workers a particular type of gas chromatographic system was constructed. The main feature of the system is the incorporation of a simplified "equilibration" blood-gas extraction and injection unit. The system is used to enable the oxygen, carbon dioxide and nitrous oxide contents of blood to be quantified with accuracy and with rapidity.

METHOD

Hydrogen, which has been thoroughly dried by passage through a Molecular Sieve drier, is used as the carrier gas. This divides into two streams both passing through separate needle valves and thence to a katharometer, in one case via an inert comparison column, and in the other case via an active column complex. The latter complex comprises a sampling valve, a phosphorus pentoxide drier and two adsorption columns linked in parallel. The adsorption columns are maintained at room temperature. Attached to the sampling valve is a disposable retention-equilibration [illegible]

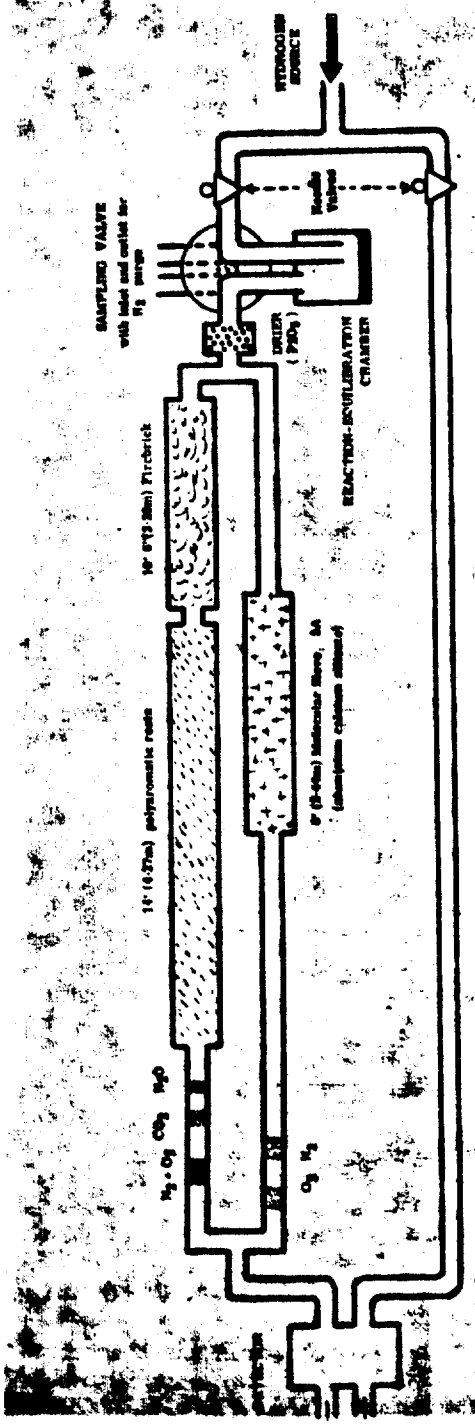

FIG. 2
Schematic diagram of apparatus.

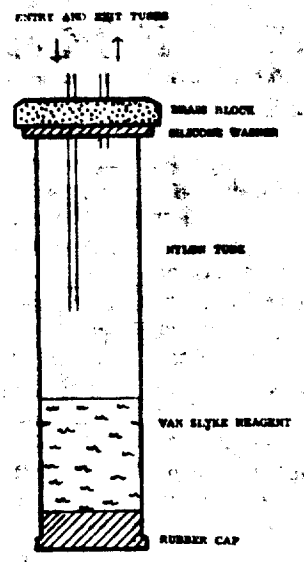

FIG. 3
The reaction-equilibration chamber (diagrammatic).

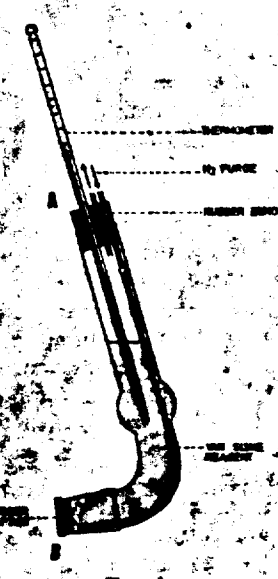

FIG. 4
The reagent purger for degassing Van Slyke reagents.

a purging gas line, this having adjustable taps at its entry and exit ports. The response of the katharometer is displayed on a 1 mV chart recorder (fig. 2).

Analyses are carried out by injecting 80 μl. blood into Van Slyke reagent in the reaction-equilibration chamber. The gases liberated from the blood are equilibrated between the liquid and gaseous phases present in the chamber, the gases in the latter phase being then passed on to the columns. The reaction and the equilibration are conducted at room temperature. The gas contents of the blood are determined by comparing the peak heights for blood with the peak heights produced by known amounts of these gases. The calibration for carbon dioxide is carried out by using carbonate solution, for oxygen by using room air, and for nitrous oxide by using cylinder nitrous oxide.

FIG. 5
The reaction-equilibration chamber assembly attached to the agitator, the reagent purger and the sampling valve. Blood for analysis is being injected into the chamber.

Extraction and injection unit.

The equilibration extraction and injection unit used is a development of one originally devised by B. Warren* (1967, personal communication). A detailed description of the apparatus is to be given elsewhere (Davies and Askill, 1970). Essentially it consists of a small disposable chamber made up from a nylon tube of length 50 mm (F&M 9855.001) closed at its lower end by means of a rubber cap (F&M 8251.001) and pressure-sealed at its upper end by being brought into contact with a thick silicone rubber washer (F&M 5080.5022). The chamber so formed is surrounded and held in place by a cradle which screws on to a block holding the washer. The volume of the chamber is 1.3 ml. Metal entry and exit tubes (19 s.w.g.) pierce the block and washer to enter the chamber from above. The entry tube extends 22 mm into the chamber so that it terminates approximately 13 mm above the level of the liquid present during extraction. The exit tube extends 1 mm into the chamber. The whole chamber assembly is attached to an agitator arm and is capable of being vigorously agitated up and down (figs. 3 and 5).

The metal entry and exit tubes from the chamber are connected by nylon tubes (length 60 cm) to a gas sampling valve (F&M G.V.11) via the entry and exit ports intended for the gas sampling loop. The carrier gas stream, which normally passes straight through the sampling valve directly to the columns can, by turning this valve, be diverted to pass through the extraction chamber, to sweep into the columns the gaseous contents of the chamber. To prevent possible contamination of the columns with water vapour from the chamber, a drier of 33 per cent phosphorus pentoxide in dry firebrick is interposed between the valve and the columns.

A separate stream of hydrogen gas, derived from a separate reduction valve, passes in a purging gas line to the sampling valve. This stream can be made to perfuse the reaction chamber to flush the chamber clear of air. The flow can be controlled by means of adjustable taps at the entry and exit ports. The exit port is connected to a water seal.

Reagent and reagent purger.

Van Slyke reagent is made up from the following two solutions (solution A must be freshly prepared).

A.	Potassium ferricyanide		1.5 g
	Saponin		0.4 g
	Octyl alcohol		1.0 ml
	Water	to	50 ml
B.	Lactic acid AR (H and W)		1.0 ml
	Water	to	50 ml.

Equal volumes of solutions A and B are mixed together. To 25 ml of the reagent, 0.6 ml of antifoam emulsion (BDH 1-2287-1) is added and the whole vigorously shaken.

Degassing of the Van Slyke reagent, prior to analysis, cannot be carried out in the reaction chamber, as the use of relatively wide-bore entry and exit tubes to and from the chamber causes liquid throw-over to become a serious problem if reagent is agitated in the chamber in a stream of gas. (Narrow-bore entry and exit tubes fitted originally to the chamber were found to be impracticable for blood analysis as they readily become obstructed with blood debris.) Consequently degassing of the reagent is carried out independently in a separate reagent purger. This arrangement proves to be advantageous in that, having the total volume of the reagent already degassed at the commencement of the session, considerable time is saved during the course of the individual analyses. In addition the reagent purger acts as a handy reservoir for the reagent.

The reagent purger (figs. 4 and 5) was constructed from a Quick-Fit drying tube (MF 23/2). At one end (A) two wide-bore needles together with a thermometer calibrated in tenths of a degree, pass through a rubber bung into the tube. The two needles act as entry and exit for hydrogen gas, the entry needle being connected to the hydrogen purge line and the exit needle to a water seal. A rigid polyethylene tube ($\frac{1}{16}$ in. bore) attached to the entry tube carries the purge gas stream to the other end (B) of the drying tube. The drying tube is clamped in such a way that it can swivel to a horizontal position thus allowing reagent to be poured into it. It is closed at B with a thin rubber septum. With the drying tube in the vertical position the hydrogen gas passes through the total volume of the reagent, effectively stripping it of all dissolved gases. The usual quantity of reagent degassed is 25 ml. Degassed reagent is removed from the purger by piercing the thin rubber septum with a needle and withdrawing the required volume into a syringe. The thermometer immersed in the reagent enables the temperature of the reagent to be monitored.

Adsorbents and column arrangements.

The solid adsorbents used for the separation of the blood-gases into their individual components are Polypak 2 and Molecular Sieve 5A. The adsorbents are packed in separate nylon columns [illegible] which are linked in parallel to stainless steel Y joints by means of Swagelok compression couplings. The columns are maintained at room temperature.

Polypak 2 is a polyaromatic resin which separates carbon dioxide and nitrous oxide. This is a relatively difficult separation and, using adsorbent of 40–80 mesh, a column length of 14 ft. (4.27 m) was required. Oxygen and nitrogen are not separated in this column and are eluted together as a composite.

Molecular Sieve 5A is an aluminium calcium silicate which, after activation by heating to a temperature of 300–400°C for several hours, will separate oxygen and nitrogen. This separation is relatively easy and the column length is not critical. Carbon dioxide and nitrous oxide are permanently adsorbed by Molecular Sieve and these gases will, in time, poison the adsorbent.

The gas flow differential between the two active columns was so adjusted that the carbon dioxide peak associated with the Polypak column and the oxygen peak associated with the Molecular Sieve column were of comparable height and could both be made to span the major part of the chart recorder paper, without the necessity of having to change the attenuation of the katharometer during the course of each analysis. The correct gas flow differential was obtained simply by lengthening or shortening the Molecular Sieve column accordingly. In this particular column arrangement, when analyzing blood with an oxygen content of approximately 20 vol. per cent and a carbon dioxide content of approximately 50 vol. per cent, the correct gas flow differential was produced when the Molecular Sieve column, packed with adsorbent of 60–85 mesh, was from 7 ft. to 8 ft. (2.13–2.44 m) in length. To prevent the overlapping of the gases eluted from the two columns it was found necessary to increase slightly the retention time of the Polypak column. This was effected by adding to that column an inert fore-column of dry powdered firebrick of length 10.5 ft. (3.20 m).

The peaks in order of appearance are oxygen (M.S.), the composite (Polypak), nitrogen (M.S.) carbon dioxide (Polypak) and finally nitrous oxide (Polypak) (fig. 6).

In the analysis of blood, the oxygen and carbon dioxide peaks, having been made to span the major part of the chart recorder paper, can be

used for very accurate quantification of oxygen and carbon dioxide contents. The nitrous oxide peaks are necessarily of lesser magnitude but are usually adequate for reasonable accuracy in the quantification of nitrous oxide contents. The nitrogen peaks obtained in the analysis of 80 μl blood samples are, however, of inadequate magnitude for the quantification of nitrogen contents; such determinations would necessitate separate analyses using much larger blood samples.

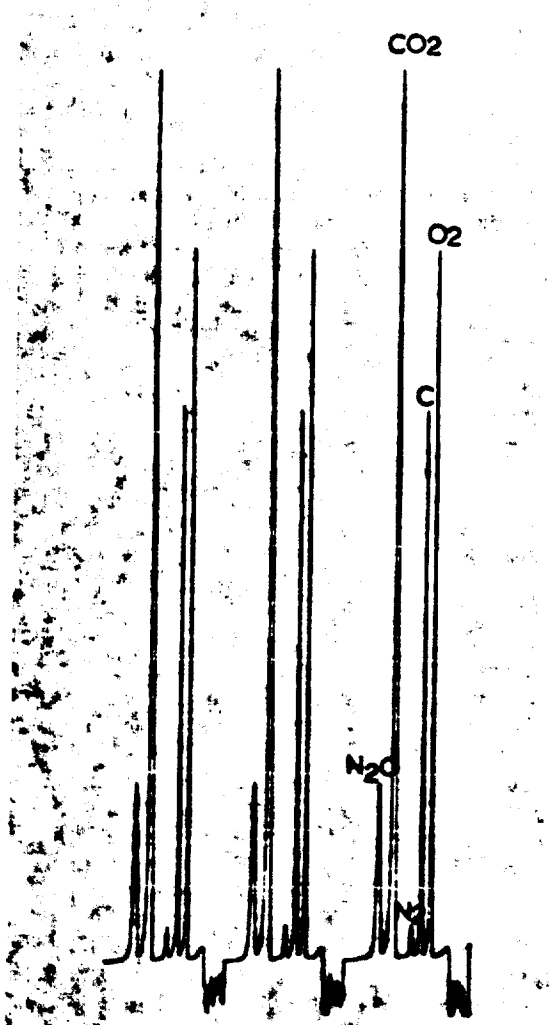

FIG. 6

Chromatogram of three identical blood samples. The peaks in order of appearance (reading from right to left) are oxygen (O_2), the composite (C), nitrogen (N_2), carbon dioxide (CO_2), nitrous oxide (N_2O).

The system of columns is not completely stable. The activity of the Molecular Sieve diminishes over a period of several weeks; the retention times of both oxygen and nitrogen become less, and the time between the appearance of the two peaks shortens. Thus this column must be replaced from time to time as reactivation in situ is not possible when hydrogen is the carrier gas and nylon the column material. Poly-pak appears to remain active almost indefinitely.

The total rate of flow of hydrogen carrier gas in the active columns, and also in the inert comparison column, is set at 120 ml/min. This is also the rate of flow through the reaction-equilibration chamber when the chamber is incorporated into the carrier gas stream. To maintain this rate of flow a delivery pressure of approximately 1.8 kg/cm² is needed.

Detection and recording units.

The katharometer used is maintained at a constant temperature of 30°C. For most analyses, the attenuator is adjusted to full sensitivity and the bridge current usually set between 235 and 265 mA (maximum 300 mA). The signal from the detector is fed to a 1 mV chart recorder, both 6-inch and 10-inch types being used.

Preparation for analysis.

The carrier gas is turned on and the flow rates through the active column complex and through the inert comparison column equalized by adjusting the respective needle valves. The filament current is turned on and the katharometer allowed to stabilize: reasonable stability is attained in approximately 1 hour.

Degassing of the reagent is usually completed in under 1 hour. When reagent blanks have demonstrated that not more than slight traces of dissolved oxygen remain in the reagent, the gas flow rate to the reagent purger is considerably reduced.

It was found that vigorous purging of the reagent could reduce its temperature slightly. If the reagent temperature is found to be more than a quarter of a degree Centigrade below the ambient temperature, then the reagent has to be warmed by holding the purger in the hand for a

for seconds until these temperatures become the same.

Reagent blanks are carried out as follows. A disposable reaction-equilibration chamber is assembled and flushed with hydrogen gas from the purging gas line for 30 seconds. Van Slyke reagent is removed from the reagent purger by means of a 1 ml tuberculin syringe, the reagent being withdrawn and voided back into the chamber several times before a volume of 200 μl is taken, a spacer being used to ensure accurate reproducibility of the volume. The reagent is introduced into the chamber through the rubber cap sealing its lower end. Further flushing of the chamber with hydrogen is carried out for 60 seconds. The purging gas line entry and exit ports are then closed and the chamber agitated for 50 seconds. When agitation has been terminated the gaseous contents of the chamber are injected on to the chromatographic columns. The injection is timed to last 10 seconds.

Preparation of blood samples.

Blood for analysis is drawn into reservoir syringes, the deadspaces of which have been filled with heparin solution (1000 units/ml). The capacity of the reservoir syringe should be at least 1 ml, this being the minimum volume of blood needed to carry out analysis in triplicate. As the present study was exclusively of a comparative nature, relatively large volumes of blood were used, the capacities of the reservoir syringes being 10 ml and 20 ml.

A correction will normally have to be made for the error produced by filling the deadspace of the syringe with heparin solution. In the present study, because of its comparative nature, such corrections were, obviously, not necessary.

The syringes are sealed with plastic caps and, whilst being continuously kept in motion on a mechanical rotator, the contents are allowed to attain room temperature. Before analysis the plastic cap is removed and replaced by a thin rubber cap.

For analysis, blood is transferred from the reservoir syringe to a 100 μl syringe. The microsyringe has to be filled anaerobically and in such a way that the deadspace of the syringe and needle, which can amount to an appreciable proportion of the total volume of the syringe, does not produce inaccuracies in the volume and in the concentration of the blood samples. To obviate this difficulty the following technique can be used: the plunger of the microsyringe is removed and the needle of this syringe then thrust through the rubber cap of the reservoir syringe; the pressure in the reservoir syringe is raised so that a stream of blood is made to run through the microsyringe in a retrograde direction, effectively flushing out the needle and barrel; the plunger of the microsyringe is reinserted into the barrel against the moving stream of blood; finally the microsyringe is detached from the reservoir syringe and excess blood in the former voided slowly with the syringe held in a vertical position. To prevent air contamination of the needle end, the needle is not wiped. A needle with a bore of 25 s.w.g. is used.

Analysis of blood.

The procedure carried out is identical with that used for reagent blanks except that, immediately after the chamber and the reagent in the chamber have been effectively purged with hydrogen gas, 80 μl of blood is injected slowly and carefully, through the rubber chamber cap, into the reagent. During injection of the blood the entry port of the purging gas line is closed but the exit port left open until the injection has been completed. Chamber agitation, and injection of the gaseous contents of the chamber on to the chromatographic columns are, as in the case of reagent blanks, timed to last 50 seconds and 10 seconds respectively.

Duplicate analyses of each blood sample are usually undertaken. If, however, the peaks in the duplicates differ in height by more than 1 per cent, then a third analysis is carried out. The time taken to carry out individual analyses is 4 minutes.

After the completion of the analysis of each blood sample the room temperature and the reagent temperature are noted and recorded.

Calibrations and calculations.

Calibration for carbon dioxide is carried out by injecting 80 μl potassium carbonate solution of known strength into a chamber containing the standard volume of degassed reagent (200 μl). The technique of equilibration and injection of

CALIBRATION OF BLOOD GAS CHROMATOGRAPH

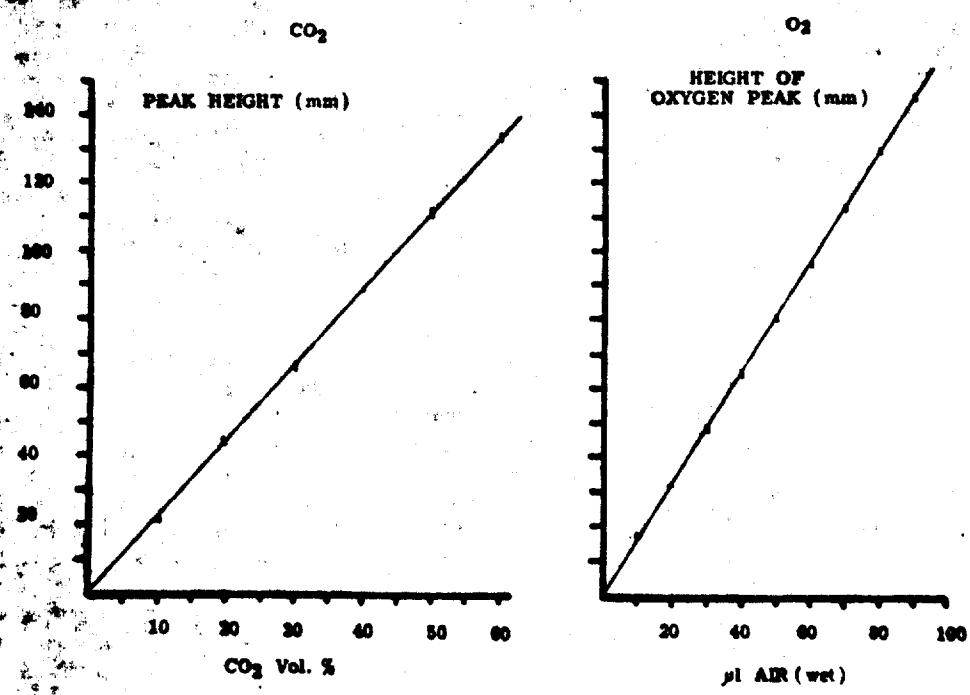

FIG. 7

Relationships between the height of the carbon dioxide peak and the amount of carbon dioxide liberated in the reaction-equilibration chamber from the potassium carbonate solution (on left) and between the height of the apparent oxygen peak and the amount of air injected into the chamber (on right). Peak height determinations were carried out in triplicate for each of the different values of carbon dioxide and air; each one of the three results is plotted separately.

the gaseous contents on to the columns is identical with that used in blood analysis.

As it was found that the katharometer response, measured as peak height, was linearly related to the amount of carbon dioxide liberated from carbonate solution (fig. 7), only one strength of carbonate solution is required for calibration purposes. The strength of solution selected for routine use was one equivalent to 50 vol. per cent carbon dioxide. The standard carbonate solution is made up by accurately weighing the appropriate amount of pure anhydrous potassium carbonate (which, immediately before weighing, has been thoroughly dried in an evacuated desiccator) and dissolving it in boiled distilled water. Taking the gramme-molecular volume of carbon dioxide to be 22.26 l., the weight of potassium carbonate needed to make up 1 litre of the required solution is 3.1040 g.

Because of the high solubility of carbon dioxide, the equilibration of the gas between the gas and liquid phases in the equilibration chamber is markedly affected by changes in temperature. It was found, therefore, necessary to keep room and reagent temperatures reasonably constant. Such temperatures are monitored with thermometers graduated in tenths of a degree. A change of temperature by as much as a quarter of a degree Centigrade necessitates recalibration for carbon dioxide.

The carbon dioxide content of the blood (CO_2) is calculated as follows:

[illegible]

where D_{blood} is the height of the carbon dioxide peak produced by the blood sample and $D_{K_2CO_3}$ is the height of the carbon dioxide peak produced by a potassium carbonate solution of strength equivalent to 50 vol. per cent carbon dioxide.

Calibration for oxygen is carried out by injecting 80 μl of air saturated with water vapour into a chamber containing degassed reagent. The technique of equilibration and injection of the gaseous contents on to the columns is again identical with that used in blood analysis.

It was found that the response of the katharometer, measured as the height of the apparent oxygen peak, bore an exactly linear relationship to the amount of air injected (fig. 7). Therefore only one standard volume of air is needed for calibration purposes.

The microsyringe used for injecting the air should contain a small quantity of water in order to saturate the air with water vapour. Care must be taken to avoid warming the syringe when handling it. When injecting the air into the chamber the exit port of the chamber must be kept open so that, during the course of the injection, the pressure inside the chamber remains the same as the ambient pressure.

The volume of reagent used in the above calibration should be 280 μl in order that the ratio between gas and liquid volumes in the reaction-equilibration chamber is equal to that present during analysis of blood. It is necessary, therefore, to carry out an additional reagent blank using 280 μl of reagent. However, because of the low solubility of oxygen in water, it was found that little appreciable error was produced by using the standard volume of reagent (200 μl).

Two corrections have to be applied when using room air for oxygen calibration. First, the volume of moist air injected at ambient temperature and pressure must be expressed as a volume of dry air at standard temperature and pressure. The relevant volume correction factor is found from standard tables. Secondly, a correction has to be made for the presence of argon in the air. Room air contains approximately 0.94 per cent of argon and, as this argon is eluted along with the oxygen fraction, the apparent oxygen peak produced by the injected air will be appreciably greater in height than the peak that would be produced by the injection of the equivalent volume of pure oxygen. In calculating the argon correction factor, cognizance must be taken of the fact that the katharometer response to argon is not the same as the katharometer response to an equal volume of oxygen, as the thermal conductivities of the two gases differ. This difference of katharometer response was determined by using a sampling loop to deliver equal volumes of the two gases to the chromatograph. The argon-to-oxygen katharometer response ratio, when measured as peak height, was found to be 1.039 to 1. From this it was calculated that the argon correction factor was 0.956.

Because of the low solubility of oxygen in water, the equilibration in the reaction-equilibration chamber is little affected by changes in temperature. Frequent calibrations are, therefore, not necessary.

The oxygen content of the blood (Co_2) is calculated as follows:

$$Co_2 = (D_{blood} - D^1_{blank}) \times \frac{F_{vt} \times 20.95}{(D_{air} - D^2_{blank}) \times F_A} \text{ vol. per cent}$$

where D_{blood} is the height of the oxygen peak produced by the blood sample, D^1_{blank} is the height of the oxygen peak produced by the 200 μl reagent blank, D_{air} is the height of the apparent oxygen peak produced by air injected for calibration, D^2_{blank} is the height of the oxygen peak produced by 280 μl reagent blank, F_{vt} is the volume correction factor needed to reduce the volume of air saturated with water vapour at ambient temperature and pressure to that of dry air at STP, and F_A is the argon correction factor (0.956).

Calibration for nitrous oxide is carried out by injecting 80 μl nitrous oxide into the reaction-equilibration chamber, equilibrating, and then injecting the gaseous contents on to the columns. It was found that the response of the katharometer measured as peak height bore an exactly linear relationship to the amount of nitrous oxide injected; therefore, as in the case of oxygen calibration, only one standard volume of nitrous oxide is used. The nitrous oxide is obtained from a cylinder and is injected as dry gas. The volume is corrected to that at standard temperature and pressure.

Sampling of nitrous oxide is carried out by means of a microsyringe having a gastight plunger. The syringe is filled by a technique similar to that used for blood sampling: nitrous oxide is passed through the syringe in a retrograde direction effectively flushing it clear of air, the plunger being replaced against the moving stream of gas.

When injecting nitrous oxide into the chamber it was found impossible to avoid completely contamination of the gas with room air. With the technique used the contamination amounted to approximately 1 per cent of the volume injected. This has to be allowed for in calculating the nitrous oxide content of blood.

It was found essential, in this calibration, to keep the ratio between gas and liquid volumes in the reaction-equilibration chamber equal to that present during analysis of blood. Therefore 280 μl of reagent is used.

The nitrous oxide content of the blood (C_{N_2O}) is calculated as follows:

$$C_{N_2O} = D_{blood} \times (F_{vd} \times 100/D_{N_2O}) \text{ vol. per cent}$$

Where D_{blood} is the height of the nitrous oxide peak produced by the blood sample, D_{N_2O} is the height of the nitrous oxide peak produced by the injected gas sample, and F_{vd} is the volume correction factor needed to reduce the volume of dry gas at ambient temperature and pressure to that of dry gas at STP.

RESULTS

Reproducibility studies.

Katharometer response (measured as peak height) was determined in 15 consecutive analyses of a standard carbonate solution, the analyses being carried out at constant room temperature. The coefficient of variation of the peak height was 0.25 per cent. The detailed results are given in table I.

Ten consecutive analyses of one blood sample were carried out. The analyses were completed in approximately 1 hour, during which time little appreciable change should have occurred in the gas contents of the blood. Standard deviations and coefficients of variation for oxygen, carbon dioxide and nitrous oxide were calculated. The coefficients of variation for the three gases were, in each case, approximately 0.5 per cent. The detailed results are given in table II.

TABLE I

Peak heights, measured in millimetres, produced fifteen consecutive analyses of a standard carbonate solution carried out at constant room temperature.

143.0	143.3	143.5
143.3	143.0	143.7
143.0	143.3	143.3
143.0	143.5	143.7
143.3	142.3	143.0

SD 0.35 mm. Coefficient of variation: 0.25%.

TABLE II

Results of ten consecutive analyses of one blood sample carried out by gas chromatography.

	Oxygen content (vol. %)	Carbon dioxide content (vol. %)	Nitrous oxide content (vol.
	16.34	51.99	14.0
	16.34	51.66	14.0
	16.25	51.90	13.9
	16.15	51.54	14.0
	16.26	51.63	14.0
	16.20	51.31	13.8
	16.23	51.41	14.0
	16.11	51.96	13.9
	16.23	51.96	13.8
	16.10	51.96	14.0
Mean value (vol. %)	16.22	51.73	14.00
SD (vol. %)	0.084	0.255	0.07
Coefficient of variation (%)	0.52	0.49	0.5

Comparison with results obtained by Van Slyke technique (carried out in collaboration with D. G. L. Wood).

Oxygen and carbon dioxide contents of twelve blood samples were simultaneously determined by the gas chromatographic method and by the Van Slyke manometric method. Duplicate analyses were carried out by each of the two methods. The coefficients of correlation of the gas chromatographic estimations and the Van Slyke estimations were +0.999 in the case of oxygen and +0.995 in the case of carbon dioxide. The regression equations relating the estimations by the two respective methods were:

Oxygen

$$GC\ (O_2) = 1.017\ (\text{Van Slyke } O_2) - 0.15 \text{ vol. \%}$$

Carbon dioxide

$$GC\ (CO_2) = 0.982\ (\text{Van Slyke } CO_2) + 1.17 \text{ vol. \%}$$

The results are presented graphically in figure 4.

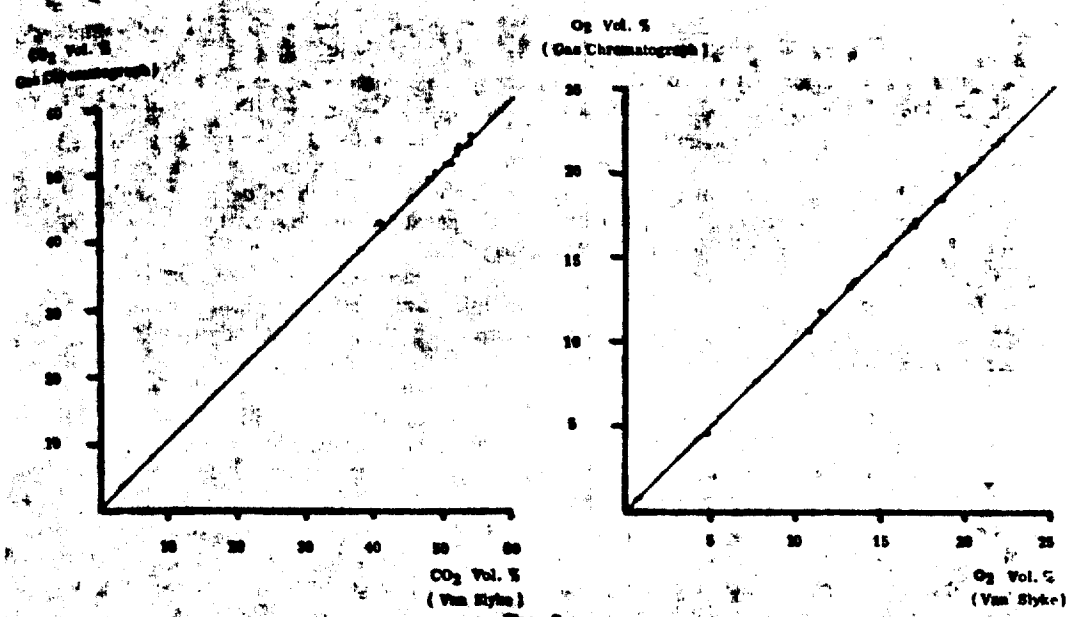

FIG. 8

Comparisons of carbon dioxide and of oxygen contents of twelve blood samples simultaneously determined by gas chromatography and by the Van Slyke manometric method.

DISCUSSION

The gas chromatographic system described displays marked consistency of response during analysis. Fifteen consecutive analyses carried out at constant room temperature of a standard carbonate solution produced peaks whose heights had a coefficient of variation of 0.25 per cent. Reproducibility studies conducted on blood samples are complicated by greater sampling errors than is the case with a simple carbonate solution; nevertheless ten consecutive measurements of oxygen, carbon dioxide and nitrous oxide contents of a single blood sample were carried out with a coefficient of variation of approximately 0.5 per cent for each of the three gas contents analysed.

Values for oxygen and carbon dioxide contents of blood obtained by the gas chromatographic method were compared with values obtained by the Van Slyke method. There was very close agreement of the values obtained by the two methods.

The amount of blood needed for analysis is small. As only 80 μl is injected into the chromatograph, a 1-ml sample of blood withdrawn from the patient is adequate for carrying out analysis in triplicate. The fact that the amount of blood needed for analysis is small is an important consideration when performing serial blood analyses, especially on children as well as on laboratory animals.

The technique of analysis is rapid; duplicate blood analyses, carried out by a single-handed operator can be completed in 8 minutes. The reaction-equilibration chambers are each assembled in a few seconds and, because they are disposable, analytical work is not interrupted by the need to wash apparatus. In practice, the chambers were washed and used again, but, as a supply of 200 was available, washing was undertaken after the analyses had been completed. Time is also saved by having the total volume of the Van Slyke reagent degassed at the commencement of the analytical session.

For accuracy in analysis the equilibration method of extraction and injection would seem to be superior to other methods. In the equilibration method a concentrated slug of gas is injected rapidly and consistently on to the columns. The peaks produced are very sharp and reproducible and therefore quantification of the individual

gases can be carried out as accurately by the measurement of peak height as by the measurement of peak area. It is important in the method that the volume of reagent introduced into the chamber is accurately measured, as the ratio between the gas and liquid volumes in the chamber must be exactly the same in all the analyses. A disadvantage of the method is that the distribution of the more soluble gases between the two phases is affected by changes in temperature. This problem could be overcome by temperature-regulation of the equilibration chamber (Hamilton, 1962) but this would add very greatly to the complexity of the apparatus. It was decided, therefore, to rely upon careful monitoring of the ambient temperature and of the temperature of the reagent, and recalibrating for carbon dioxide and for nitrous oxide whenever significant changes occurred in these temperatures.

Gas leaks from the reaction-equilibration chambers must be prevented. To this end the silicone rubber washer closing the upper end of the chamber must be changed fairly frequently. The rubber cap through which liquids and gases are introduced into the chamber will, because it is located at the bottom of the chamber, be effectively sealed with a layer of liquid, and thus gas leaks through it should not occur.

It was found that bubbling of the blood-reagent mixture during the injection of the blood-gases on to the columns, caused considerable errors in the results obtained. It is important, therefore, to use an effective antifoam agent during the analysis.

If the gas chromatograph is to be used single-handed then the use of adsorption columns linked in parallel is advantageous because, as has been stated, these can be so arranged that the need to alter the sensitivity of the katharometer during the course of the analysis is obviated. The relevant column lengths given may not be exactly applicable to all similar column arrangements as the techniques of column packing vary, and different brands of adsorbents do not always have identical adsorption efficiency. It was found that, provided the Molecular Sieve column was changed when its efficiency became diminished, the column arrangement was very stable with respect to its integrative function.

A meticulous technique in blood sampling is important. Particular attention has to be directed to the elimination of errors due to deadspace when filling the microsyringe. Another source of error is the inclusion of microbubbles of air in the injected blood; fortunately, however, air contamination can always be detected, as the nitrogen peak produced during analysis is of increased magnitude.

The content of dissolved nitrogen in blood samples could be determined by this method of analysis; such determinations would be of value in investigations into ventilation-perfusion relationships in the lung. The nitrogen peak produced by the analysis of 80 μl of blood was found to be too small for the purpose of accurate quantification and, therefore, separate analyses using at least 200–300 μl of blood have to be carried out. As no reagent is required for extraction of nitrogen from the blood, the injection of a relatively large quantity of blood into the small reaction-equilibration chambers presents no special problems.

ACKNOWLEDGEMENTS

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UNE METHODE DE CHROMATOGRAPHIE GAZEUSE POUR L'ANALYSE QUANTITATIVE DES GAZ SANGUINS

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EFFECT OF NITROUS OXIDE ON THE WHITE-CELL COUNT IN LEUKEMIA

D. W. EASTWOOD, M.D.,* C. D. GREEN, M.D.,† M. A. LAMBDIN, M.D.,‡ AND RICHARD GARDNER§

CHARLOTTESVILLE, VIRGINIA

TOXICITY from nitrous oxide has been assumed to be entirely the effect of associated reduction of oxygen tension. Goldman,[1] in 1928, reported the intermittent administration of nitrous oxide to rats and mice during a two-month study. He observed no deleterious effects. The first indication that nitrous oxide might have a toxic effect to man came in 1955, when Gormsen[2] presented the results of treatment of a case of tetanus in which nitrous oxide was used along with chlorpromazine and curare. The thrombocytopenia and leukopenia occurring on the fifth day of treatment were attributed to curare.

Mollaret,[3] in the same year, treated a case of tetanus with nitrous oxide and observed a drop in white-cell count, especially granulocytes. The bone marrow was apparently normal. In 1956 Lassen and his associates[4] described 13 cases of tetanus and came to the conclusion that "it seemed possible that the acute aplastic anemia was due to prolonged nitrous oxide anesthesia." Patients were more liable to sepsis, and biopsy showed hypoplastic bone marrow with reduced erythropoiesis of a megaloblastic type. Agranulopoiesis, with a pronounced shift to the left, and numerous myeloid cells of undetermined character were present. Post-mortem examination showed pronounced bone-marrow depression, probably with totally abolished myelopoiesis. Similar reports followed.[5-8]

With this developing evidence Lassen reversed his previous suggestion that tetanus be treated with the technic of nitrous oxide analgesia and sedation. In 1957, Kieler and his associates,[9] using various concentrations of nitrous oxide, nitrogen and oxygen on embryonic mouse-heart myoblasts in tissue culture, concluded that nitrous oxide was a mitotic poison preventing interphase cells from entering mitosis and causing spindle destruction and chromosomal abnormalities in dividing cells. They stated further that "cell death occurred after treatment with nitrous oxide. The cytocidal effect was most pronounced in cultures suffering from lack of oxygen, but high oxygen tension also had cytostatic and cytocidal effects."

In November, 1959, Lassen and Kristensen[10] reported the effect of prolonged administration of nitrous oxide in chronic myeloid leukemia. In 2 patients with this diagnosis nitrous oxide administration by means of nasal catheter reduced the total number of myeloid white blood cells and produced some thrombocytopenia after five and fifteen days of treatment. The white-cell count rose rapidly after withdrawal of the nitrous oxide. The red-cell count and hemoglobin concentration were not changed. From their work no conclusion can be reached about whether the patient's life was prolonged by the therapy. The nitrous oxide inhalation did reduce the vitamin B_{12} level, which is usually elevated in chronic myeloid leukemia.

A study by Okamoto[11] of prolonged administration of nitrous oxide to rats indicates severe leukopenia after an initial leukocytosis, relative lymphocytosis, hemoglobin and red-cell decrease and decreased myeloblastic and erythroblastic activity in the bone marrow.

As a preliminary to prolonged nitrous oxide administration to human beings animal experiments were carried out in our laboratory. Studies were completed on 57 albino rats, of which 32 were control animals and 25 experimental. The animals were placed in chambers in which the temperature, humidity and gas concentrations were kept constant. Air was administered to the control animals whereas the experimental animals were exposed to 80 per cent nitrous oxide, with 20 per cent oxygen. This combination produced a state of intoxication, but the animals responded to noise and consumed slightly less food and water than the control animals. The average white-cell count of the control rats exposed to air remained at 13,000. The mean count of the experimental animals exposed to nitrous oxide dropped progressively to 1130 by the sixth day of exposure. The median test showed a differential between the control and experimental animals that was significant (p less than 0.001). Polymorphonuclear cells disappeared more rapidly from the circulating blood than lymphocytes did. However, both were reduced in number. The normal dense rat bone marrow containing little fat showed progressive hypoplasia as the duration of the nitrous oxide administration continued. Reproduction of cells apparently ceased, and mitoses disappeared.

With the knowledge that nitrous oxide was capable of depressing the white-cell count in the albino white rat it was decided to administer this gas to 2 patients in whom the diagnosis of acute myelogenous leukemia had been made and who were refractory to other forms of treatment.

CASE REPORTS

CASE 1. A 3-year-old girl was admitted to the hospital after 10 days of illness with an initial hematocrit of 19 per cent and a white-cell count of 60,000. The diagnosis of mye-

*Professor of anesthesiology, University of Virginia School of Medicine.
†Assistant professor of anesthesiology, University of Virginia School of Medicine.
‡Assistant professor of pediatrics, University of Virginia School of Medicine.
§Student, University of Virginia School of Medicine.

logenous leukemia was confirmed by bone-marrow aspiration. Therapy was initiated with antibiotics, steroids and 6-mercaptopurine, on which the white-cell count rose to 170,000. The patient was placed in a croupette, and nitrous oxide administration was started. Frequent gas samples analyzed by means of a Pauling oxygen meter and a nitrogen meter indicated concentrations of 30 to 40 per cent nitrous oxide, 20 to 30 per cent oxygen and 30 to 50 per cent nitrogen. Moderate sedation resulted from this treatment. The white-cell count after the 1st day of nitrous oxide therapy was 130,000, with a marked shift to the left. A total period of therapy of 72 hours was instituted, after which the white-cell count was reduced to 21,500. Two transfusions were administered during this time. Twenty-four hours after the nitrous oxide was discontinued the white-cell count was 20,000, and in 48 hours it was 27,600 (Fig. 1). The patient continued to show marked purpuric

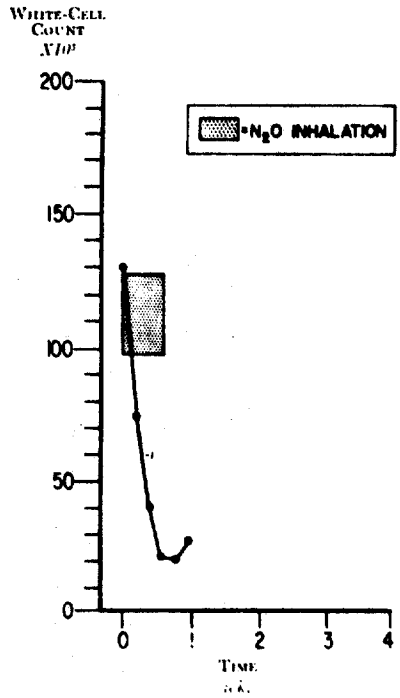

FIGURE 1. *Response of the White-Cell Count to Nitrous Oxide Inhalation in a Patient with Myelogenous Leukemia (Case 1).*

The count had averaged 135,000 during the three weeks before nitrous oxide inhalation. The gases inhaled consisted of nitrous oxide, 30 to 40 per cent, oxygen, 20 to 30 per cent, and nitrogen, 30 to 50 per cent.

lesions and died 2½ days after the discontinuance of the nitrous oxide inhalation with evidence of intracranial hemorrhage.

CASE 2. A 13-year-old boy with a 3-week history of illness was given the diagnosis of myelogenous leukemia. The white-cell count was 131,000. Severe joint pain and swelling required the use of narcotics. The pain was only partially controlled by meperidine, 75 mg. given intramuscularly every 3 hours. Nitrous oxide administered at approximately 35 per cent concentration in a croupette was started and continued for 3 days, during which the white-cell count fell to 57,000 (Fig. 2). The patient no longer needed narcotics. The nitrous oxide therapy was discontinued because of intercurrent hyperpyrexia. The patient's white-cell count then rose to 175,000. At that time nitrous oxide therapy by nasal-catheter administration was again started. Epistaxis made this method unsatisfactory, and a plastic hood fitted over the patient's head was substituted (Fig. 3). By this method it was possible to administer 60 per cent nitrous oxide, 40 per cent oxygen. Therapy was given during the night only. Relief of pain and discomfort lasted all day during this course of treatment.

The white-cell count rapidly fell, followed by a gradual decline. The count remained between 15,000 and 5000 cells for 20 days. On the 18th day of this 2d period of therapy the patient suffered a convulsive episode. The white-cell count at that time was 6500. A diagnosis of leukemic in-

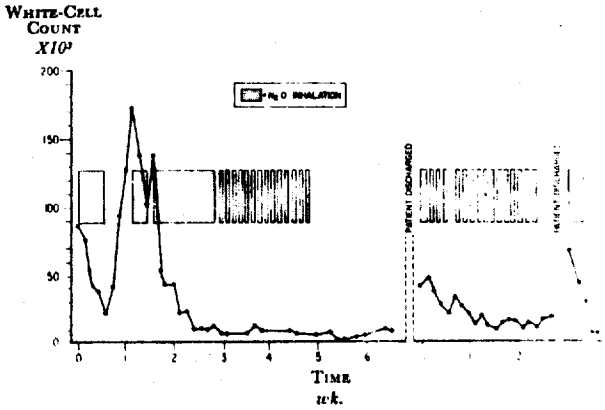

FIGURE 2. *Response of the White-Cell Count to Nitrous Oxide Inhalation in Case 2.*

The response during three hospital admissions is shown. Intermittent inhalation relieved the patient's pain and maintained the white-cell count at near normal levels.

filtration of the brain was suggested as responsible for this episode. Nitrous oxide was discontinued. Three days later the white-cell count dropped to 1250, and the hematocrit to 18 vol. per cent. The patient was given blood transfusions, with a good response, and was discharged from the hospital 1 week later, free of pain and ambulatory and with a white-cell count of 10,300.

One month later he was readmitted to the hospital for evaluation, and the white-cell count was found to be 29,100. Pain had recurred. Nightly nitrous oxide therapy during 19

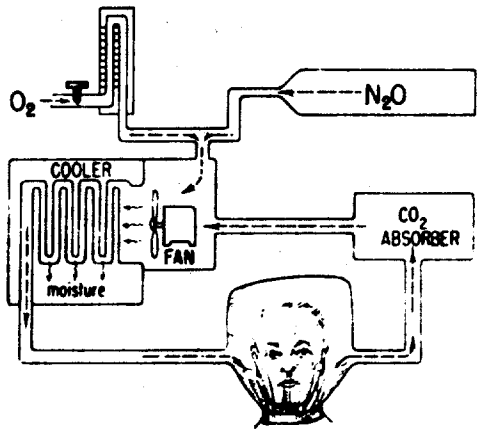

FIGURE 3. *Principle of Nitrous Oxide Administration.*

A soft plastic hood was used over the patient's head for administration of inhalation therapy. The oxygen was obtained from a wall source, and the nitrous oxide from a tank supply. Cooling and circulation of gas was effected by a modified unit from an oxygen tent. Maintenance of pressure at approximately 2 mm. of mercury above ambient pressures minimized inboard leaks. The flows used were 6 liters of nitrous oxide and 4 liters of oxygen per minute.

days of a 3-week hospital stay reduced the white-cell count to 12,200, and he was discharged again. During this admission leukemic infiltration was demonstrated by x-ray ex-

amination in a number of areas, including the left shoulder and the right hip, knee and ankle.

Readmission in 2 weeks because of joint pain and swelling revealed a white-cell count of 36,300 that rose to 80,200 by the 3d day of admission. Thirty-six hours of nitrous oxide administration produced a rapid fall to 6400. Approximately 10 days after this admission the patient began to bleed from the mouth and showed gross hematuria and blood in the stool. He continued to run a high fever and had a progressive downhill course until he died.

The possibility that the effect observed both in the cases and in the animal study was due to contaminants of nitrous oxide must be considered. This possibility has been carefully investigated, and we believe it to be unlikely.[12]

Discussion

The results of the use of nitrous oxide in the management of tetanus by Lassen and his associates[4] and others,[2,3,5-8] the bone-marrow depression observed in the laboratory animal and the reduction of white-cell formation in acute and chronic myelogenous leukemia have led to the conclusion that nitrous oxide is toxic on chronic administration. The mechanism by which nitrous oxide produces depression in cell formation is not known but may be similar to that causing depression in the central nervous system, inducing analgesia, anesthesia and unconsciousness. Though it is doubtful whether nitrous oxide will be of major benefit in the management of leukemia it is hoped that studies of the action on the hematopoietic system may reveal information concerning the mechanisms of narcosis.

An impressive aspect of the use of nitrous oxide inhalation observed was the immediate relief of pain, which continued even while the patient was receiving nitrous oxide only during the sleeping hours, but recurred after he left the hospital. Immediate relief was experienced upon resumption of nitrous oxide therapy. This patient's insistent request for "the bag over my head" on each of two readmissions raised the question of addiction.

Summary and Conclusions

Nitrous oxide inhalation effectively depresses the white-cell formation in the albino white rat. The effect of nitrous oxide inhalation is also present in myelogenous leukemia, and 2 cases are presented in which this effect is demonstrated. Further elucidation of the mechanism by which bone-marrow depression occurs may help explain the mechanism of anesthesia produced by nitrous oxide.

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CLAIMS OF SAFETY FOR NITROUS OXIDE (BARRING ANOXIA) ARE REPLETE IN THE LITERATURE, INCLUDING THIS PUBLICATION. WHILE THE MATERIAL PRESENTED BY DR. GREEN DOES NOT RESTRICT USEFULNESS IN CLINICAL ANESTHESIA, AS HE SO STATES, THE EFFECT OF PROLONGED NITROUS OXIDE ADMINISTRATION IS INCLUDED SINCE IT REPRESENTS A NEWLY-DISCOVERED PHARMACOLOGIC ACTION. WHETHER USEFUL IN MANAGEMENT OF MALIGNANT DISEASE OR BY CONTRIBUTION TO KNOWLEDGE OF THE MECHANISMS OF ACTION OF ANESTHETIC DRUGS WILL BE DECIDED IN THE FUTURE.

The opinion has gradually arisen that nitrous oxide in the absence of hypoxia produces analgesia and a state of hypnosis without any toxic manifestations. The lack of potency which removed it from the list of life-threatening agents was interpreted as proof of its benign nature. Until recently, few studies searching for toxic manifestations of prolonged nitrous oxide inhalation were conducted. Goldman[9] repeatedly administered nitrous oxide, ethylene, and acetylene in oxygen to rats and mice for periods of up to two months. He reported, in 1928, that no deleterious effects were observable after anesthetization every other day for periods averaging one hour.

A study of nitrous oxide as a possible bacteriostatic agent was reported by Benigno[1] in 1941. Benigno found that coliform bacterial growth was inhibited by nitrous oxide only upon the complete exclusion of oxygen and at pressures of 28 to 50 atm.

DISCOVERY OF TOXICITY

Considerations of this kind led Bjorniboe and his associates[2, 14] to study nitrous oxide as an analgesic in the treatment of severe tetanus. These workers stated that nitrous oxide produced a mild narcosis and no harmful side-effects. In the four cases which they reported in 1953 it was recognized that certain manifestations of toxicity were displayed, but these were ascribed to one or more of the associated drugs which the patients received or to the primary disease. During the following two years, several other cases of toxicity following prolonged nitrous oxide administration were reported,[3, 10, 17, 19, 24] and, in 1956, Lassen[13] published a report concerning the recognition of the role of nitrous oxide in producing granulocytopenia and bone marrow depression. Subsequently, several additional reports[21, 23] of bone marrow depression due to nitrous oxide inhalation have appeared.

Lassen's study included 13 cases of tetanus. Six of these patients received nitrous oxide for prolonged periods and subsequently developed granulocytopenia accompanied in several instances by thrombocytopenia. The seven tetanus patients who received no nitrous oxide did not develop granulocytopenia, though a large assortment of other drugs was given. By review of the cases it was possible to demonstrate that hematologic complications had appeared after three to four days of nitrous oxide inhalation. Bacter-

emia and myocarditis were late accompaniments of the bone marrow depression, but it seems impossible to establish the causal relationships between the myocarditis and the bacteremia, nitrous oxide, or tetanus.

The hematologic picture of several patients was studied in some detail. On the third to the fifth day after the beginning of continuous inhalation of 50 per cent nitrous oxide the white cell count would drop abruptly to levels of 2000 to 4000 cells per cu. mm. of blood. The bone marrow, which had been previously normal or consistent with severe infection, would show megaloblastic erythropoesis and changes of granulocytes closely resembling those of pernicious anemia. The onset of thrombocytopenia was inconsistent, occurring as late as 12 days after the onset of leukocytopenia. Treatment with vitamin B_{12} and other drugs was without evident benefit. If nitrous oxide was continued, the degree of bone marrow destruction increased. After discontinuance of nitrous oxide, incipient remission would appear in the bone marrow in three to four days.

IN VITRO STUDIES

A study into some aspects of the nature of the cellular depression produced by nitrous oxide was reported by Kieler[13] in 1957. Heart tissue from embryonic mice was grown in culture and exposed to varying concentrations of nitrogen, oxygen, and nitrous oxide. The inhibition of growth was recorded, and microscopic study of the cells was carried out. From these studies, Kieler concluded that nitrous oxide was "a mitotic poison, preventing interphase cells from entering mitosis and causing spindle destruction and chromosonal abnormalities . . . "

A number of investigators have obtained differing results in attempting to ascertain the effects of nitrous oxide on cellular metabolism. Bülow and associates[4] did not find an inhibition of oxygen consumption when guinea pig brain homogenate was exposed to an 80 per cent nitrous oxide, 20 per cent oxygen mixture. In a study which differed in a number of aspects, including the use of guinea pig brain slices rather than homogenate, Quastel[25] found that nitrous oxide did inhibit oxygen consumption. Bülow[4] was unable to confirm Quastel's findings. Subsequent studies by Pittinger[20] and by Levy[15] also failed to detect reduction in metabolism of guinea pig brain homogenate as the result of exposure to nitrous oxide, but a study by Hosein[12] revealed reduction of oxygen consumption in both homogenate and slices of rat brain. The latter author and associates concluded from their studies of the hydrogen transport system that cytochrome b of the succinoxidase system and the flavoproteins, diaphorase and TPN-cytochrome c-reductase, were sensitive to nitrous oxide.

The effects of 90 per cent nitrous oxide and 10 per cent oxygen on a number of fungi were studied by Schreiner.[22] The introduction of nitrous oxide over the culture caused an abrupt cessation of growth of hyphae. The oxygen consumption of the colony decreased. By photomicrographs

it was shown that protoplasmic movements through the cells ceased, and intracellular agglomerates appeared. Returning the colony to a nitrogen-oxygen gas mixture caused the agglomerates to disappear, protoplasmic movements to be resumed, and growth of the cell to commence at the same rate as previously. However, in each colony a certain percentage of cells appeared to have been irreversibly affected.

ANIMAL STUDIES

In our laboratory, albino rats exposed to 80 per cent nitrous oxide, 20 per cent oxygen show a rapid response, so that the median white-cell count in six days will be less than 10 per cent of normal.[11] The granulocytes disappear from the blood stream more rapidly than do the lymphocytes. Platelets are still found in the peripheral blood, but morphologically they appear abnormal. The mortality rate between the completion of the fifth and sixth days of exposure is 50 per cent.

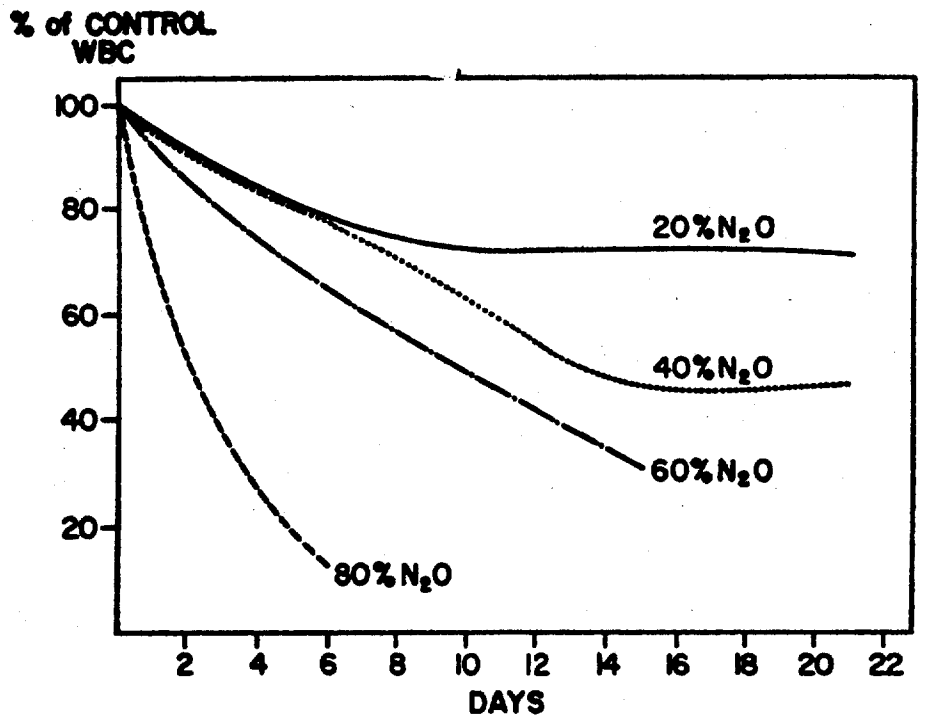

Figure 1. The response of the peripheral blood white-cell count of the white rat when exposed to nitrous oxide is shown. The gas mixtures used were nitrous oxide 20 per cent, oxygen 20 per cent, nitrogen 60 per cent; nitrous oxide 40 per cent, oxygen 20 per cent, nitrogen 40 per cent; nitrous oxide 60 per cent, oxygen 20 per cent, nitrogen 20 per cent; and nitrous oxide 80 per cent, oxygen 20 per cent.

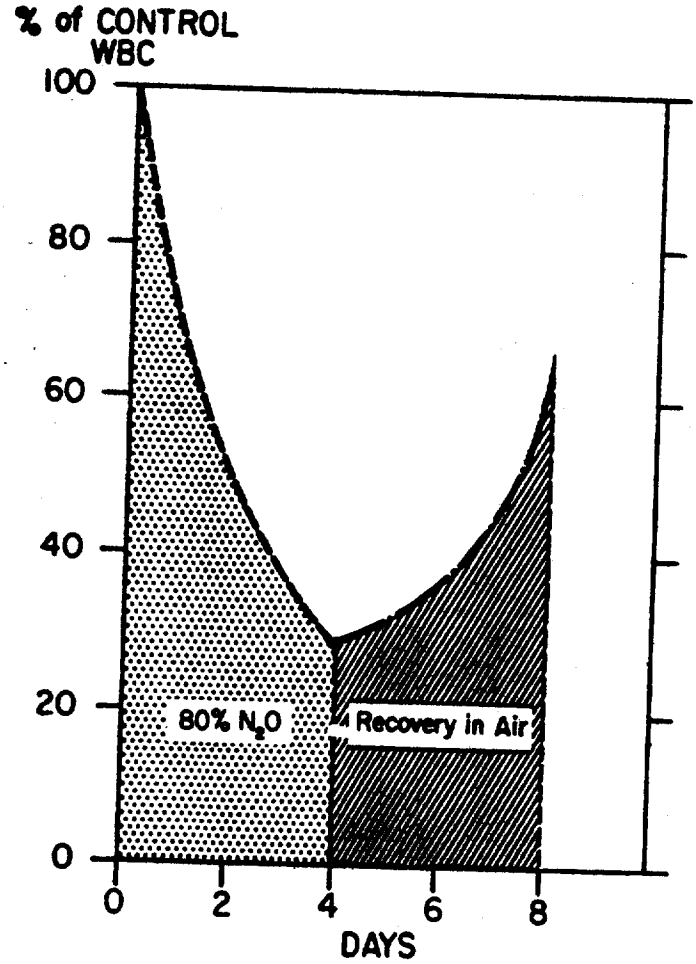

Figure 2. The recovery of the white-cell count of the white rat after four days' exposure to 80 per cent nitrous oxide, 20 per cent oxygen is shown. The recovery atmosphere was air.

The bone marrow of the rats showed a progressive hypoplasia as exposure to nitrous oxide was prolonged. Reproduction of cells apparently ceased, and mitoses disappeared. The only cell form which appeared unaffected was the megakaryocyte. Intracellular bacteria were demonstrated.

Further investigations in our laboratory have shown that 60 per cent nitrous oxide, 20 per cent oxygen, and 20 per cent nitrogen is still toxic to the rat bone marrow, and the peripheral white cell count drops to one third of normal in 15 days. Forty per cent and 20 per cent nitrous oxide, each in 20 per cent oxygen, appear to cause a reduction of white cells in the peripheral blood, but after three weeks of continuous exposure the animals appeared healthy, and their counts remained over 50 per cent of normal (Fig. 1).

Animals exposed to 80 per cent nitrous oxide, 20 per cent oxygen for four days showed an ability to recover rapidly (Fig. 2). Two days after being returned to room air atmosphere the white-cell count rose slightly (from 25 per cent of normal to 35 per cent), and after four days in room air the white-cell count was rapidly returning to normal (65 per cent of control).

The information available strongly suggests that one of the most apparent effects of nitrous oxide is the inhibition of cell growth or reproduction. This may be of importance in eventual explanation of the findings of Ebert[7]—that nitrous oxide protects single cells against oxygen-dependent radiation damage—and the demonstration by Evans[8]—that nitrous oxide will protect the mouse against total body irradiation.

TREATMENT OF LEUKEMIA

Soon after the toxicity of nitrous oxide was recognized, Lassen[16] reported the effects of the prolonged administration of this gas to two patients suffering from chronic myeloid leukemia. The gas, administered by nasal catheter for five and 15 days, reduced the total number of myeloid white blood cells and reduced the thrombocyte count without alteration of the red-cell count or hemoglobin concentration. The vitamin B_{12} level, which is usually elevated in myeloid leukemia, was reduced. The white-cell count

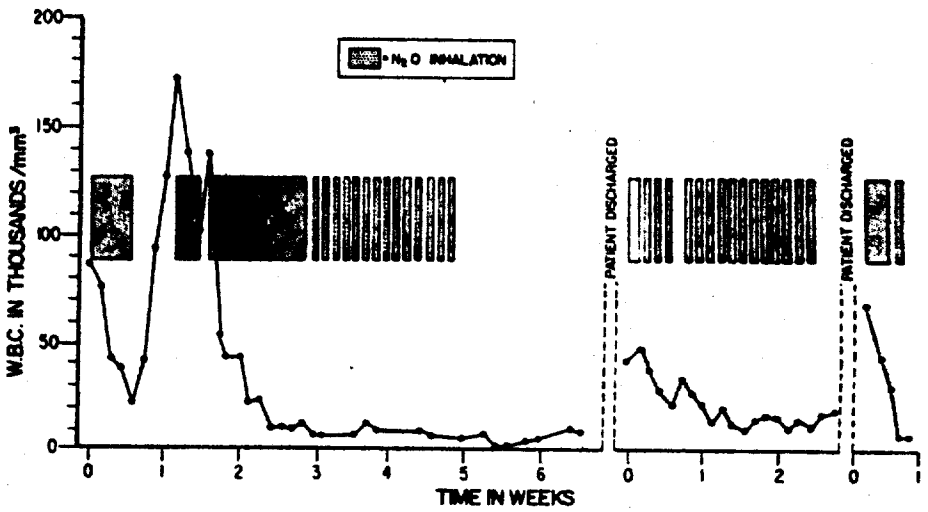

Figure 3. The response of the white-cell count of a boy having myeloid leukemia is shown. Intermittent inhalation of nitrous oxide-oxygen mixtures during three hospitalizations resulted in depression of the white-cell count and relief of pain. (From Eastwood, D. W., Green, C. D., Lambdin, M. A., and Gardner, R.: Effect of nitrous oxide on the white-cell count in leukemia. New Eng. J. Med. 268:297, 1963.)

rose rapidly after discontinuance of the nitrous oxide. As the authors pointed out, it is impossible to determine if the inhalation therapy altered the course of the disease.

In our clinic, two children having myeloid leukemia were treated by nitrous oxide inhalation.[6] In both patients the white-cell count was rapidly lowered from an excess of 100,000 cells per cu. mm. of blood to near normal levels (Fig. 3). In one instance, definite x-ray evidence of progressive bone destruction was obtained while the white-cell count was being maintained below 15,000 cells per cu. mm. of blood. Although each patient had thrombocytopenia and repeated hemorrhage, it is impossible to determine if the nitrous oxide aggravated or influenced this event.

Again, it is impossible to determine if the nitrous oxide favorably affected the course of the disease. In one case, the analgesic properties of the gas were most effective, producing excellent pain relief from the agony of long-bone destruction.

SUMMARY

Nitrous oxide will depress the bone marrow and the peripheral white-cell counts of man and animals after prolonged administration. Cell studies and molds have been used in in-vitro systems to extend these findings. The depressant effects of the gas have been utilized in the treatment of leukemia, but no evidence is available to show that life was prolonged. The analgesic properties were most useful.

There is no evidence that any toxic property of nitrous oxide becomes manifest during its use as an anesthetic agent in less than 48 hours. There is no evidence that any alteration is necessary in the use of nitrous oxide as an anesthetic for surgical procedures.

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Toxicity Anesth., Proc. Res. Symp. 1967: 114-122. 1968.

10

Cloid D. Green, M.D.

The Effect of N_2O on Ribonucleic and Deoxyribonucleic Acid Content of Rat Bone Marrow and Thymus*

The reported depressive effect of nitrous oxide on the hemopoietic system of rats (1) has been confirmed by Parbrook (2, 3). Further studies in our laboratories have shown marked variation in sensitivity of different strains of rats with accompanying differences in the nucleotide content in the cells of the bone marrow and thymus. In the course of several years, the rat colony from which individuals were obtained for the original study in our laboratory was exterminated because it was a mixed strain colony including the progeny of many white rat-wild rat crosses. The white rats which were tested in our early studies were alert and vicious.

Following extermination of the colony, our studies were continued using Sprague-Dawley rats of 150 to 250 g. It became apparent that these animals did not respond to nitrous oxide exposure in the same manner as the original animals, so an investigation of strain sensitivity to nitrous oxide was undertaken. At the same time, examination of the content of the ribonucleic acid (RNA) and the deoxyribonucleic acid (DNA) in the bone marrow and thymus was carried out.

METHODS

The animals studied were two strains of rats, the Sprague-Dawley and Long-Evans. They were confined in their usual vivarium cages which in turn were placed in an air-tight metal and Plexiglas container which could be controlled for temperature while gas mixtures were being circulated through it. In this study either air or a mixture of 70% nitrous oxide, 20% oxygen and 10% nitrogen was used at a total flow of 10 liters per min. Frequent sampling of the gases revealed less than 1% variation from the desired gas percentages and less than 0.5%

* This work was partially supported by United States Public Health Service Research Grant HE 08159.

carbon dioxide. After 8 days', exposure to one of the two gas environments, the animals were sacrificed by a brief cyclopropane anesthesia, removal of blood and excision of the heart. A white blood count and differential count were performed. Bone marrow and thymus were obtained for fixation and microscopic examination and for analysis of RNA and DNA content.

The procedure for extraction and separation of RNA and DNA was that described by Ogur and Rosson (4). The RNA content of the tissue was determined by the orcinol technique described by Ceriotti (5). The concentration of the DNA was determined by the diphenylamine reaction proposed by Dische (6). The color reactions were in each case measured in a spectrophotometer. The content of RNA and DNA was calculated in micrograms per milligram of fresh tissue.

RESULTS

A total of 30 Sprague-Dawley white rats was examined. Seventeen of these were control animals and 13 were experimental. The control animals had white blood counts averaging 5620 cells per cu mm, while the experimental animals had white blood counts averaging 4450 cells per cu mm. This reduction of 20% in the mean count was not statistically significant at the 5% level of confidence (table 10.1). Both groups of animals had normal differential white blood counts of 70 to 75%

TABLE 10.1

Comparative data of the effects of 70% N_2O on the white blood cell counts of Sprague-Dawley and Long-Evans rats

Control animals (C) of both strains were exposed to air while experimental animals (E) of both strains were exposed to N_2O (70%), O_2 (20%) and N_2 (10%). The Fisher exact probability test (7) was used for analysis of the data because white blood counts are not normally distributed and hence preclude the use of a routine parametric test. The Fisher test is nonparametric (does not assume a normal distribution) and is applicable to discrete data which have been arbitrarily dichotomized into such categories as male-female, yes-no, experimental-control or above-below the median. The data are arranged in a 2 by 2 contingency table. The probability of a combination of events can be calculated or determined by inspection of appropriate tables.

Sprague-Dawley

	−	+	
C	10	7	17
E	7	6	13
	17	13	

$p > 0.05$

Long-Evans

	−	+	
C	0	14	14
E	18	0	18
	18	14	

$p < 0.005$

lymphocytes. The Long-Evans animals had an average of 6200 white blood cells per cu mm in the 14 control animals, while the 18 experimental animals had remaining only 25% of this number, or 1470 cells per cu mm. (fig. 10.1). The difference is significant at a level less than 0.005 (7). The differential counts of the control Long-Evans animals were normal while the experimental animals had differential counts revealing 95% lymphocytes.

The bone marrow of the Sprague-Dawley animals remained normal in both control and experimental groups. The Long-Evans control animals had normal bone marrows but the experimental group showed moderate degeneration.

Table 10.2 summarizes the findings of RNA and DNA content of thymus and bone marrow. The Sprague-Dawley animals had an average of 3.8 μg of RNA per mg of thymus tissue. The DNA content averaged 22 μg per mg. Following exposure to 70% nitrous oxide for 8 days, the RNA content was statistically unchanged, averaging 4.1 μg per mg, while the DNA content was statistically reduced, averaging 15.5 μg per mg. The Long-Evans strain of animals, on the other hand, showed markedly higher RNA content in the control animals. The RNA content averaged 7.8 μg per mg, while the DNA content was 22.2 μg per mg. Following exposure to 70% nitrous oxide for 8 days, the RNA content of the Long-Evans animals increased to 14.9 μg per mg, a highly significant change. The DNA content, on the other hand, decreased to 13.2 μg per mg, also highly significant.

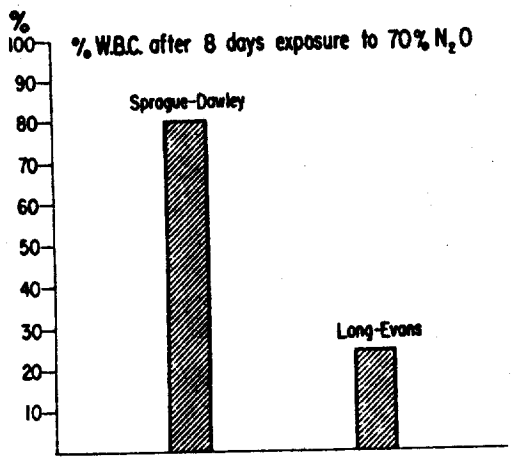

Figure 10.1. The response of the white blood cell count of adult Sprague-Dawley and Long-Evans rats is shown. After 8 days' exposure to 70% nitrous oxide, 20% oxygen and 10% nitrogen, no statistical change in the white blood cell count or differential count of Sprague-Dawley animals had occurred. Under identical testing, Long-Evans strain animals suffered a 75% decrease in white blood cell count ($p < 0.005$) and the percentage of lymphocytes increased from a normal of 72% to 95%.

TABLE 10.2

*The effect of 8 days' exposure to 70% N_2O on RNA and DNA content of organs in Sprague-Dawley and Long-Evans rats**

Strain	Content in			
	Thymus		Marrow	
	RNA	DNA	RNA	DNA
	μg/mg		μg/mg	
Sprague-Dawley				
Control	3.8 ± 0.987	22.0 ± 9.6	12.5 ± 5.83	29.4 ± 11.36
N_2O treated	4.1 ± 0.835	15.5 ± 4.23†	8.9 ± 4.45	12.7 ± 5.81†
Long-Evans				
Control	7.8 ± 2.01	22.2 ± 4.83	24.9 ± 6.72	22.8 ± 2.78
N_2O treated	14.9 ± 7.13‡	13.2 ± 7.76§	26.5 ± 11.21	10.3 ± 3.29§

* Results are expressed as mean values and standard deviation of the mean.
† $p < 0.02$.
‡ $p < 0.01$.
§ $p < 0.001$.

The bone marrow changes of the two strains of animals followed similar patterns. The control Sprague-Dawley animals had an RNA content of 12.5 μg per mg and a DNA content of 29.4 μg per mg. Following exposure to the nitrous oxide, the animals showed an RNA content of 8.9 μg per mg, a change which is not statistically significant. The DNA content, on the other hand, decreased to an average of 12.7 μg per mg, a highly significant change. The Long-Evans animals had bone marrow containing 24.9 μg per mg of RNA and 22.8 μg per mg of DNA while in the control state. Following exposure to nitrous oxide the RNA content was unchanged at 26.5 μg per mg, while the DNA content had dropped to 10.3 μg per mg, a highly significant change.

DISCUSSION

In this series of experiments, 70% nitrous oxide, combined with 10% nitrogen and 20% oxygen, was used in the treatment of the experimental animals in order to reduce the weight loss that occurred in animals exposed to 80% nitrous oxide and 20% oxygen. The weight loss observed in animals on the higher concentration of nitrous oxide appeared to be due to both dehydration and reduced food intake. When maintained on the 70% nitrous oxide mixture the animals did not become dehydrated and maintained their weights well.

The original studies of the effects of nitrous oxide inhalation on hemopoiesis in rats were carried out with a mixed strain albino colony. A considerable amount of wild rat interbreeding with the colony had occurred, although the animals used in the study were white. They

were active, aggressive and even vicious. The average white blood count found in the control animals was 15,500 white blood cells per cu mm. The animals were very sensitive to nitrous oxide, their blood showing only 1300 white blood cells per cu mm after 6 days' exposure to 80% nitrous oxide and 20% oxygen.

A pure Sprague-Dawley strain of animals, on the other hand, is represented by a quiet and docile animal. The initial white blood count of a 150- to 250-g individual of the strain ranges from 4000 to 6000 white blood cells per cu mm. Likewise, the RNA and DNA ratio in the thymus and marrow is quite low, suggesting a low metabolic activity in the organs. The Long-Evans animals, on the other hand, are a black and white colored strain. They are active and aggressive animals, although not vicious as are the rats which have a wild strain in their breeding. The white blood counts of the Long-Evans animals average approximately 1000 higher than the Sprague-Dawley, and there is a higher RNA/DNA ratio in the thymus and bone marrow, bespeaking greater metabolic activity.

The response of the two strains to 70% nitrous oxide is quite different. The Sprague-Dawley animals showed no change in their white blood counts after exposure for 8 days to 70% nitrous oxide. There was no statistical difference in the averages of 5620 and 4450 found in the control and experimental groups, respectively. Sections of the bone marrow of the Sprague-Dawley animals showed no difference in the cellularity or the apparent presence of mitotic figures, although the exact mitotic index was not determined. The differential counts of the animals after 8 days' exposure to 70% nitrous oxide remained within the normal range.

The Long-Evans animals, on the other hand, demonstrated a drop of their peripheral white blood counts to 25% of normal after 8 days of exposure to the 70% nitrous oxide mixture. The differential count had changed so that 95% of the cells remaining were lymphocytes. Sections of the bone marrow revealed moderate degeneration.

The thymus is the most important site of lymphocyte production in the rat. Microscopic studies of the thymus had shown no quantitative changes. Qualitatively, the stroma was a bit more prominent in the cortical areas; however, the medullary areas seemed to remain unchanged in size or density. Mitotic indices were not determined. Chemical analysis of the RNA and DNA content of the thymuses of both strains of animals showed marked similarity in the DNA content of the control animals and similarities in the changes produced by exposure to nitrous oxide. The initial value of 22 μg of DNA per mg of fresh tissue found in the control Sprague-Dawley rats was matched by a finding of 22.2 μg of DNA per mg of fresh tissue in the Long-Evans

rats. The DNA content of both groups of animals dropped, to 15.5 μg in the Sprague-Dawley rat and to 13.2 μg in the Long-Evans rats. The RNA content of the thymuses, on the other hand, was quite different, being 3.8 μg of RNA per mg of tissue in the Sprague-Dawley and more than twice that, 7.8 μg of RNA per mg, in the Long-Evans strain. Following exposure to nitrous oxide, the RNA content of the Sprague-Dawley rats was statistically unchanged, averaging 4.1 μg per mg, while in the Long-Evans rats the RNA content had increased to 14.9 μg per mg. The RNA/DNA ratios found were 0.173 in the control Sprague-Dawley rats, increasing to 0.275 in the experimental group. This was an increase of 53%. The Long-Evans strain, on the other hand, had an initial RNA/DNA ratio of 0.352 which increased to 1.14 in the experimental groups. This is an increase of 220%.

Similar patterns were found in the bone marrows of the two strains of animals. The DNA content of the Sprague-Dawley strain of animals was 29.4 μg per mg. In the Long-Evans animals it was 22.8 μg per mg. After exposure to nitrous oxide the DNA content of both groups dropped, to 12.7 in the Sprague-Dawley animals and to 10.3 in the Long-Evans. The RNA content of the Sprague-Dawley animals' bone marrow was statistically unchanged, averaging 8.9 μg per mg in the experimental animals; it was also unchanged in the Long-Evans animals, averaging 26.5 μg per mg. The ratios of RNA/DNA in the Sprague-Dawley animals were initially 0.423, changing to 0.70 (an increase of 65%) after exposure to nitrous oxide. The initial RNA/DNA ratio in the Long-Evans animals was 1.1, changing to 2.57 after exposure to nitrous oxide, an increase of 134%.

The similarity of DNA values in the thymus and bone marrow of the two strains suggests a nearly equal number of cells per milligram of fresh tissue. However, the elevated RNA content of the organs of the Long-Evans animals suggests a greater metabolic activity. The response of the organs to nitrous oxide administration was uniformly a decrease in DNA content, which suggests a decrease in cell numbers and the population of nucleated cells, or possibly a decrease in mitotic activity (that is, a decrease in the rate of new cell formation). Bergel (8) has pointed out that DNA is metabolically very stable and only increases in amount by anabolic processes accompanying some phase of cell division (*i.e.*, mitosis). Although ordinarily a rise in RNA content relative to the DNA present suggests a greater metabolic activity, there is the possibility that the change in the RNA/DNA ratio represents an abnormal cellular activity in some way similar to that which is sometimes seen when other methods or agents are used to interfere with metabolism.

Thymine starvation of colonies of *Escherichia coli* results in sup-

pression of DNA synthesis while RNA and proteins accumulate. The bacteria lose the ability to multiply (9). Methionine starvation, on the other hand, arrests both DNA and protein synthesis, but RNA still accumulates (10). Various antibiotics, having a variety of mechanisms of action, inhibit DNA metabolism but have lesser effects on RNA and protein metabolism. Examples are the mitomycins and porfiromycin (11), nalidixic acid and griseofulvin (12). Chloromycetin, although probably not specifically interacting with DNA, still produces an elevation of the RNA/DNA ratio in suspensions of *E. coli* (13).

Complete data concerning the sensitivity of the hemopoietic systems of a number of strains of rats will be presented in a separate publication.

SUMMARY

Adult (150 to 250 g) rats of different strains show variation in the sensitivity of their hemopoietic systems to nitrous oxide. The Sprague-Dawley strain can live in 70% nitrous oxide for 8 days without suffering a decrease in the peripheral white blood count or a change in the proportion of granulocytes to lymphocytes. Adult (150 to 250 g) Long-Evans rats demonstrate a higher initial white blood cell count, which drops to 25% of normal after 8 days in 70% nitrous oxide. The differential count will then show a predominance of lymphocytes.

Analysis of the RNA and DNA content of the thymus and bone marrow of the two strains shows a small effect on the organs of the Sprague-Dawley animals, while a more marked alteration in the RNA/DNA ratio occurred in the Long-Evans animals. In both strains the DNA content tended to decrease while the RNA content remained unchanged or increased.

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DISCUSSION AFTER PRESENTATION

Dr. Van Dyke: At the 1967 Federation meeting* a group of workers at NIH reported that carbon tetrachloride interfered with protein synthesis and that it did so by specifically interfering with the messenger-RNA synthesis (1).

Dr. Aldrete: What age were your Sprague-Dawley rats, Dr. Green?

Dr. Green: They weighed 150 to 200 g; they were larger than yours.

Dr. Aldrete: Approximately 2 or 3 months old? The disparity between your results and mine could be explained on the basis of size. We are using 50- to 60-g rats, which is immediately after weaning. In these animals the bone marrow is quite active and so the thymus, I suppose, is active too. It would be interesting to repeat the experiments with animals of this size.

Dr. Green: I suspect that if we in some way stimulated the Sprague-Dawley animals to form white blood cells, we could perhaps demonstrate the action of nitrous oxide on the bone marrow.

Dr. Chenoweth: There have been several papers lately on the effect of ether on salmonellosis in rats, and I have several letters from veterinarians who are tinkering with the use of anesthetics as antiviral agents, particularly in distemper in dogs. This is perinent perhaps to your last comment about their being something like antibiotics. There may be more there than we thought.

Dr. Brauer: I am most interested in these data. It seems to me that none of us has talked about recovery after these agents. I wonder whether we have any data on this. I have a suspicion that recovery is very rapid. If so, this provides us with a preparation that has great biochemical interest.

Dr. Green: The animals of the original colony type were exposed to 80% nitrous oxide for 4 days, at which time the white blood count was down around 30% or so of normal. The nitrous oxide was then discontinued. Forty-eight hours later there was no statistical difference in the white blood count, but at 96 hr their white blood count was back to 70% normal and was rapidly increasing.

Dr. Fink: Dr. Green, there seem to be very interesting genetic implications in the figures in that table which ended up with Sprague-Dawley rats

* Federation of American Societies for Experimental Biology.

crossed with wild rats. The table didn't show what the effect was on wild rats as such. I wonder if you could tell us about that.

Dr. Green: I do not know, sir; I haven't caught that many wild rats. I think I am not going to.

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CHEMOTHERAPY

B. Franklin Gurney, B.S., M.S., D.D.S.

Nitrous oxide: Part two

In Part One of this series, nitrous oxide-induced hypoalgesia was mentioned as a potential valuable aid in the conduct of dental operations. This report continues the discussion.

TOXICITY

Used for the production of relative anesthesia, nitrous oxide appears to be essentially nontoxic.

Over a prolonged period of time, for example, continuously for two weeks, nitrous oxide does produce bone marrow injury coupled with a decrease in white blood cells.

ADVANTAGES

Nitrous oxide is an inexpensive, nonflammable, nonexplosive gas with a pleasant odor.

Ordinarily, it does not induce laryngeal spasm, true syncope, or an increase in blood pressure, pulse rate, respiration, or release of autogeneous epinephrine. Since muscular relaxation, if any, is minimal, no mouth prop is needed. (If mouth closes, lighten nitrous oxide supply a small amount.)

Although the patient's responses are altered, he is fully conscious at all times and capable of desired cooperation. His ability to concentrate may increase or decrease. Being in an ataractic state, his psychologic outlook is considerably altered in that fear is almost completely absent. Sights and sound in a dental office which would normally induce fear reflexes are now ignored. The role, effect, and value of time is usually lost. With careful direction, the patient can be trained to increase or decrease the depth of hypoalgesia by proper mouth or nose breathing as required.

The bite reflex and tongue reflex are diminished.

The cough reflex is not affected even though other thresholds are elevated. Numerous patient reactions involving delays are eliminated, thus increasing work efficiency 30 to 50 per cent.

Hypoalgesia is rapidly reversible, hence the patient's head is clear in 5 to 10 minutes, and he leaves the office in full command of all faculties.

There are no serious medico-legal problems.

No addiction or tolerance to nitrous oxide has been reported.

SAFETY

Nitrous oxide-induced hypoalgesia is usually safe in that it dissolves rapidly

in plasma, does not penetrate most tissues or organs, and leaves the plasma rapidly. Used with oxygen it is very difficult to produce a state of hypoxia or produce a lethal dose with the open-mouth method of administration.

PREMEDICATION

Ordinarily, premedication before hypoalgesia induction is not needed, but it may be used if desired or necessary. Barbiturates, ataractics, tranquilizers, or narcotics are common agents for premedication. Of course, nitrous oxide dosage must be adjusted accordingly.

DISADVANTAGES

The equipment needed to deliver the nitrous oxide-oxygen mixture is large and bulky.

Adequate and proper prior education must be given a patient as to what is going to happen and how he is to handle the mask. Two appointments may be needed to train the patient properly for his assigned role.

Sweating by the patient may occur, which is at most a nuisance.

The patient must not be allowed to drift from hypoalgesia (Stage 1, Plane 2) to analgesia (Stage 1, Plane 3) as this could lead to a very unsatisfactory situation. The practitioner must guess at the level of hypoalgesia and rate of induction for each new patient.

The proprioceptive reflexes for balance, posture, and walking are strongly suppressed, hence a patient must be kept in the dental chair until his head is clear and he is in full command of all of his faculties.

If the patient has to expectorate, guide his body carefully as he has poor proprioceptive control.

Use electrosurgery apparatus with great care as a fear complex can arise.

Always oxygenate the patient well before his release. Question him and make sure his head is clear (10 to 15 minutes).

PRECAUTIONS

A pressure type oxygen apparatus should be available in case of emergency.

To decrease the possibility of nausea, the patient should not have food or drink for at least three hours prior to the appointment.

During induction, question the patient constantly about his subjective sensations as they are an indication of the level of hypoalgesia.

It is important that nothing occurs during induction that is painful.

Usually it is unwise to use a rubber dam on the first appointment because of the possibility of cutting off the free flow of air to the mouth and precipitating a condition of fright (in the patient) of suffocation. Indoctrination about, and use of, the rubber dam must be done with great care on patients in nitrous oxide hypoalgesia.

It may be necessary to employ a good nasal decongestant on the patient in order to promote adequate nasal breathing.

Ordinarily, a third person should be present at all times.

With increasing partial pressure of nitrous oxide in the alveolar spaces, the gas diffuses into body hollow spaces until it reaches equilibrium with the vapor tension of the inspired gas. If there are voids in the gastrointestinal tract, and it is occluded at both ends, distension, followed by uneasiness or pain may follow. A pneumothorax may increase in volume, as may the air pocket in the middle ear (both could be painful).

During hypoalgesia, the patient may have erotic dreams. With a third person present, these usually prove to be of no consequence.

ADMINISTRATION

Induction may be done rapidly, at normal rate, or slowly. Since all patients differ in the amount of nitrous oxide

needed to produce hypoalgesia, induction nitrous oxide percentages and rate must be determined for each patient. Normally, a flow rate and percentage is used that will give hypoalgesia in one to two minutes. A common starting concentration could be 25 per cent nitrous oxide with 20 per cent oxygen, followed by a reduction to 10 per cent of nitrous oxide.

Ordinarily, there is a 60 second lag between a change in the flow rate and the patient's response.

There are no rapid changes in conditions.

Provided there is 20 per cent oxygen present in the gas mixture, there is no time limit on the period of hypoalgesia.

SUBJECTIVE SYMPTOMS

Shortly after the gas flow starts, the patient usually notices a sweet taste and definite pleasant odor. Tingling may be felt in the peripheral parts of the body, especially in the finger tips and the tip of the tongue. Euphoria sets in, and the patient may feel talkative or urged to giggle or laugh. From the latter action arose the idea and term of "Laughing Gas." Lethargy and drowsiness may follow. The voice becomes throaty and husky. To the patient, sounds seem to be in the distance. His thoughts usually focus on some situation outside of the dental office. Fully conscious and cooperative, ordinary sight (such as a hypodermic needle) and sounds (as of a dental turbine) are of no consequence to him.

Temporary amnesia can arise now and then. There may be a rushing or drumming to the ears. Sights become indistinct. Pupils may dilate slightly. The face may become flushed. The sense of touch remains unaltered.

SYMPTOMS AND REACTIONS

As the degree of hypoalgesia deepens, the response to questions becomes sluggish, and speech may become slurred, indicating the degree of hypoalgesia is a little too deep.

On occasion, the patient may shed tears, which are the result of sorrowful thoughts about situations outside the dental office. Usually, if the patient is told to think of something cheerful, the lacrimation stops.

Excess euphoria and talkativeness may respond to stern verbal warnings. Sometimes a small increase in depth of hypoalgesia will calm the patient's urge to excess talk.

A rigid mandible or loss of pupillary reflexes, or both, indicate the patient is slipping into Plane 3, Stage 1 (See Figure 1) and nitrous oxide concentration should be decreased.

If the patient moves forward, some situation has arisen severe enough to stimulate a fear reflex. Whatever the cause, it must be removed or stopped. Question the patient.

Withdrawal actions usually indicate the patient is feeling pain. It is well to ask the patient if you hurt him, and then to correct the situation.

When a patient refuses to "wake up" after oxygenation at the end of the appointment, this usually indicates the patient is under autohypnosis and does not want to return to full consciousness. Adequate oxygenation plus strong counter suggestions usually solve the problem.

A so-called "refractory patient" usually is one who has had poor psychologic preparation for nitrous oxide hypoalgesia.

PRUDENCE

This article, and its predecessor, are not to be treated as substitutes for a thorough and adequate course in nitrous oxide hypoalgesia. They are for informational purposes only.

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Glen Ellyn, Illinois 60137

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

by Robert W. Hamilton, Jr., Janis D. Cohen, Gerald F. Doebbler, Lorenzo F. Exposito, John M. King, Kent H. Smith, and Heinz R. Schreiner

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

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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.

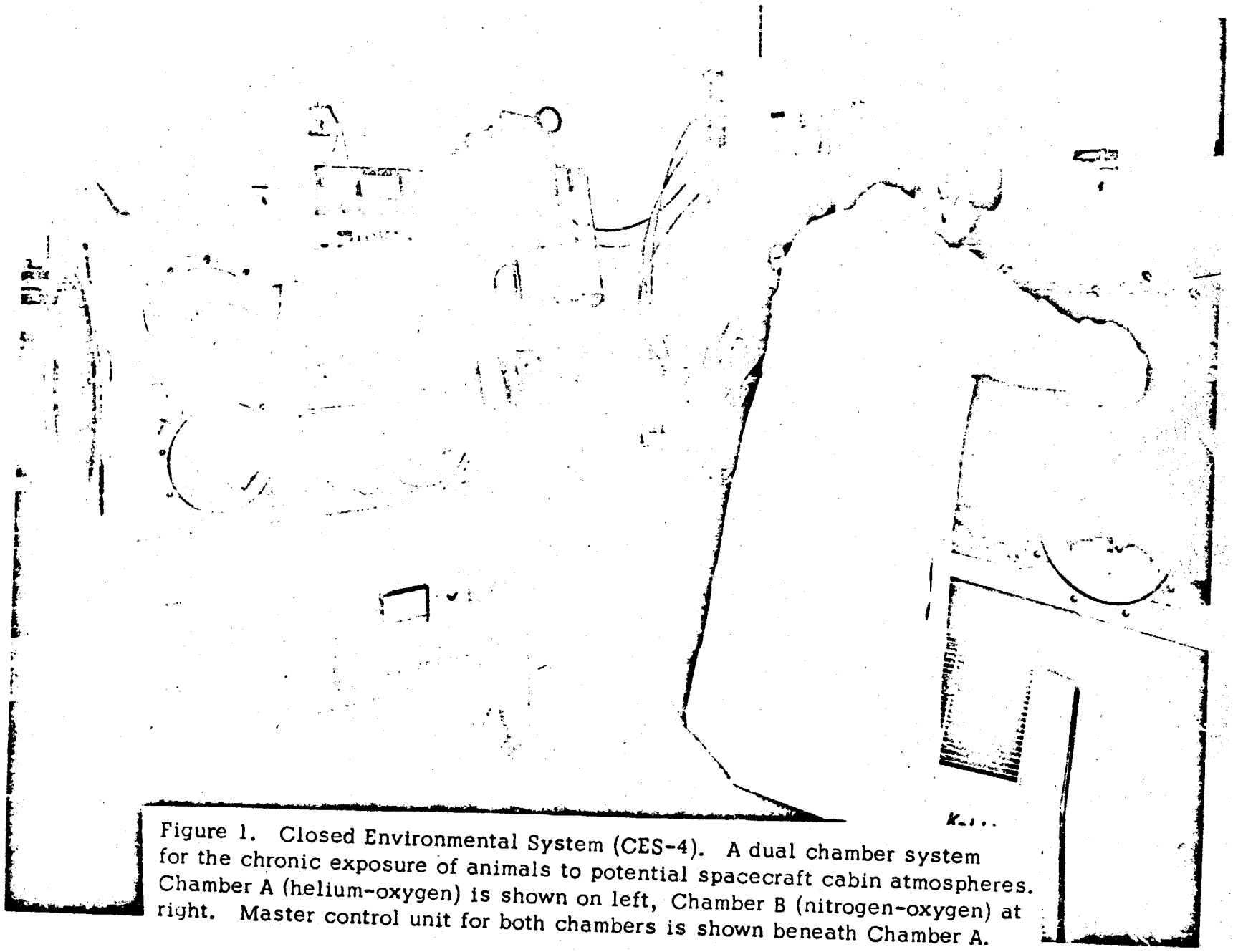


Figure 1. Closed Environmental System (CES-4). A dual chamber system for the chronic exposure of animals to potential spacecraft cabin atmospheres. Chamber A (helium-oxygen) is shown on left, Chamber B (nitrogen-oxygen) at right. Master control unit for both chambers is shown beneath Chamber A.

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:

$\underline{L}$ - lactate + NAD = pyruvate + $NADH_2$;

MDH catalyzes the citric acid cycle reaction:

$\underline{L}$ - malate + NAD = oxaloacetate + $NADH_2$;

G6PDH catalyzes the hexose monophosphate shunt reaction:

$\underline{D}$ - 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.

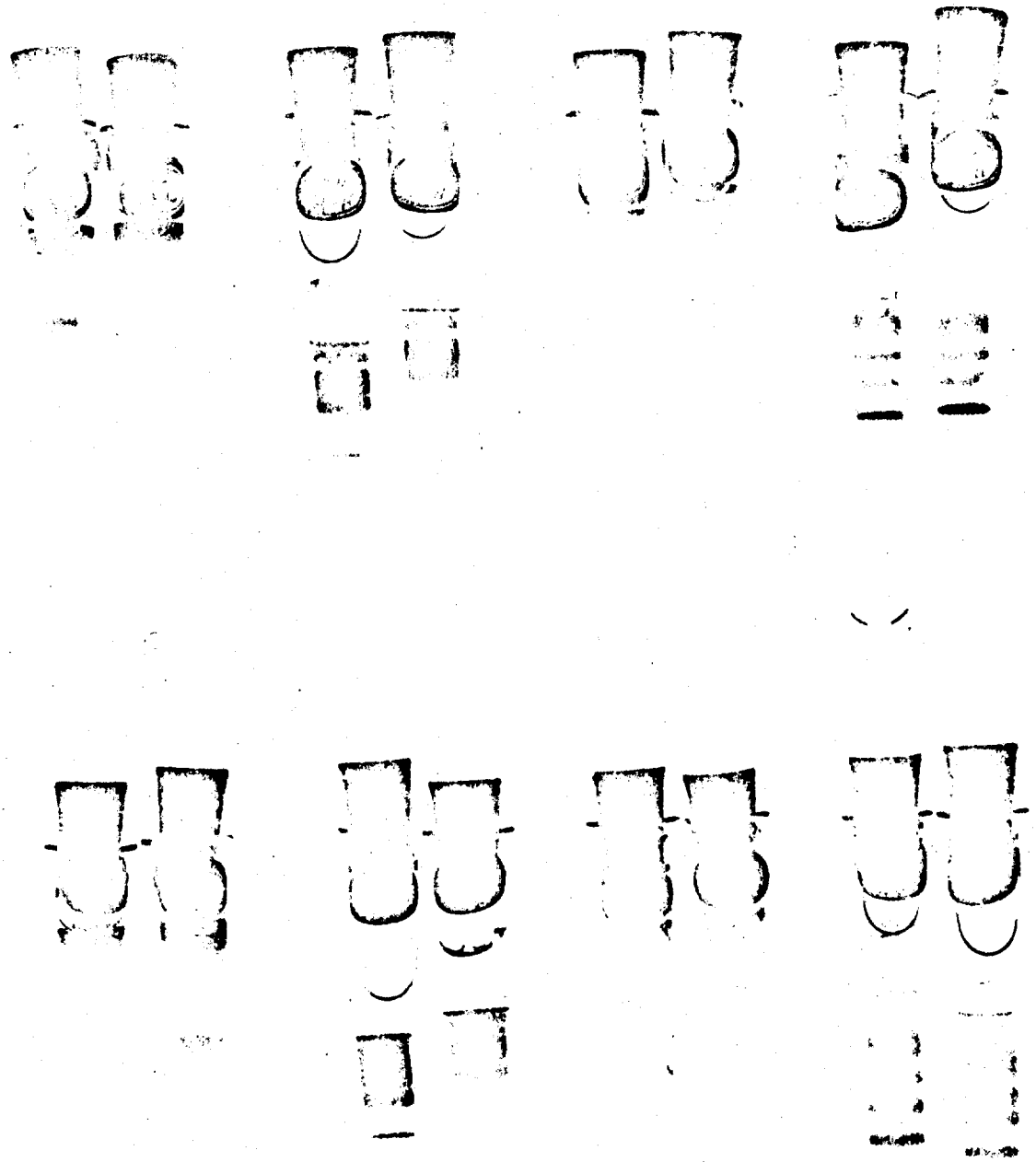


Figure 2. Photographs of Disc Electrophoresis Patterns of Mouse Muscle (M) or Heart (H) Extracts Resolved under Low (L) (pH 5.4) or High (H) (pH 8.6) pH Conditions.

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

*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

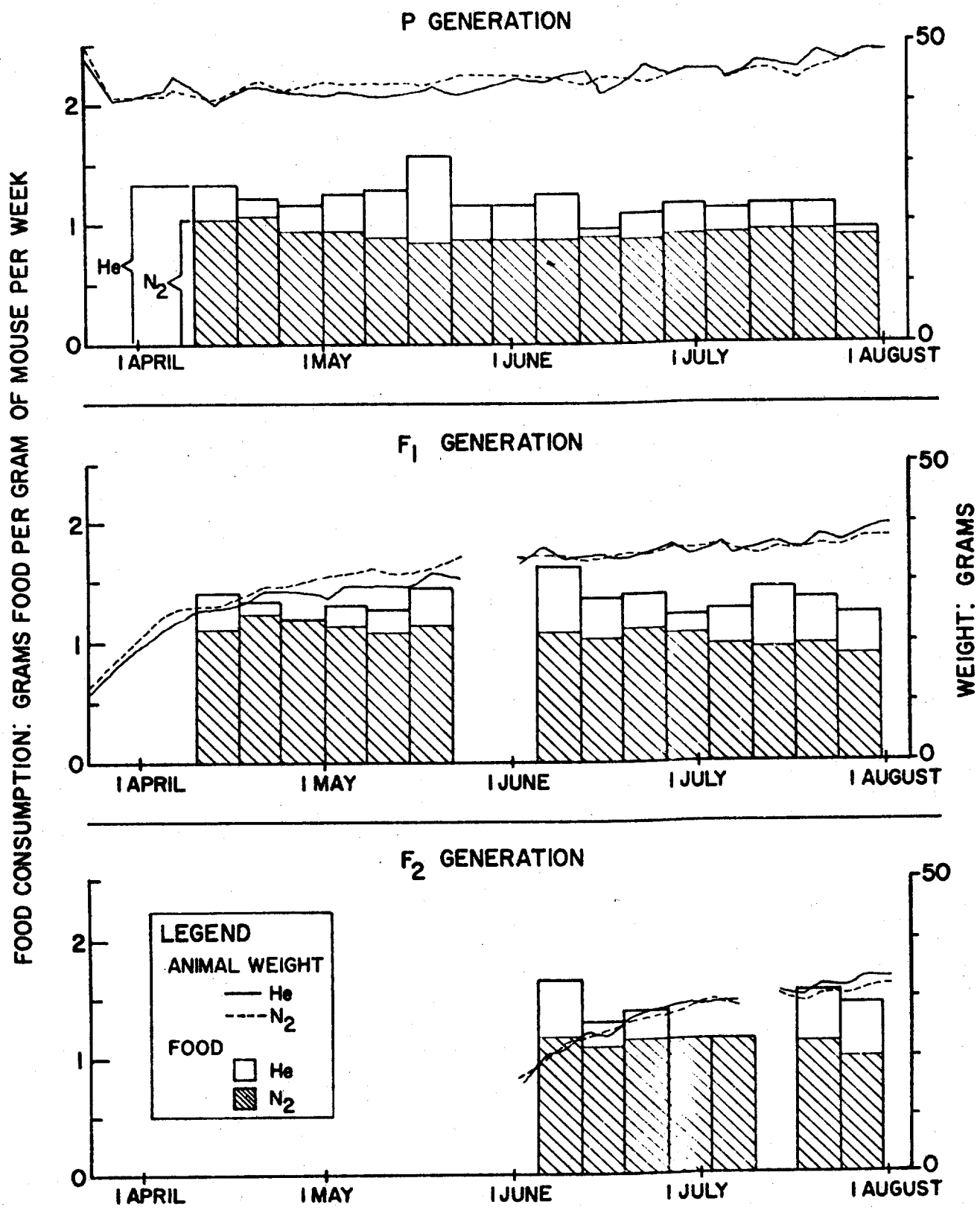


Figure 3. Food Consumption and Growth of Mice Chronically Exposed to Helium-Oxygen and Nitrogen-Oxygen at Ground Level Pressure.

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

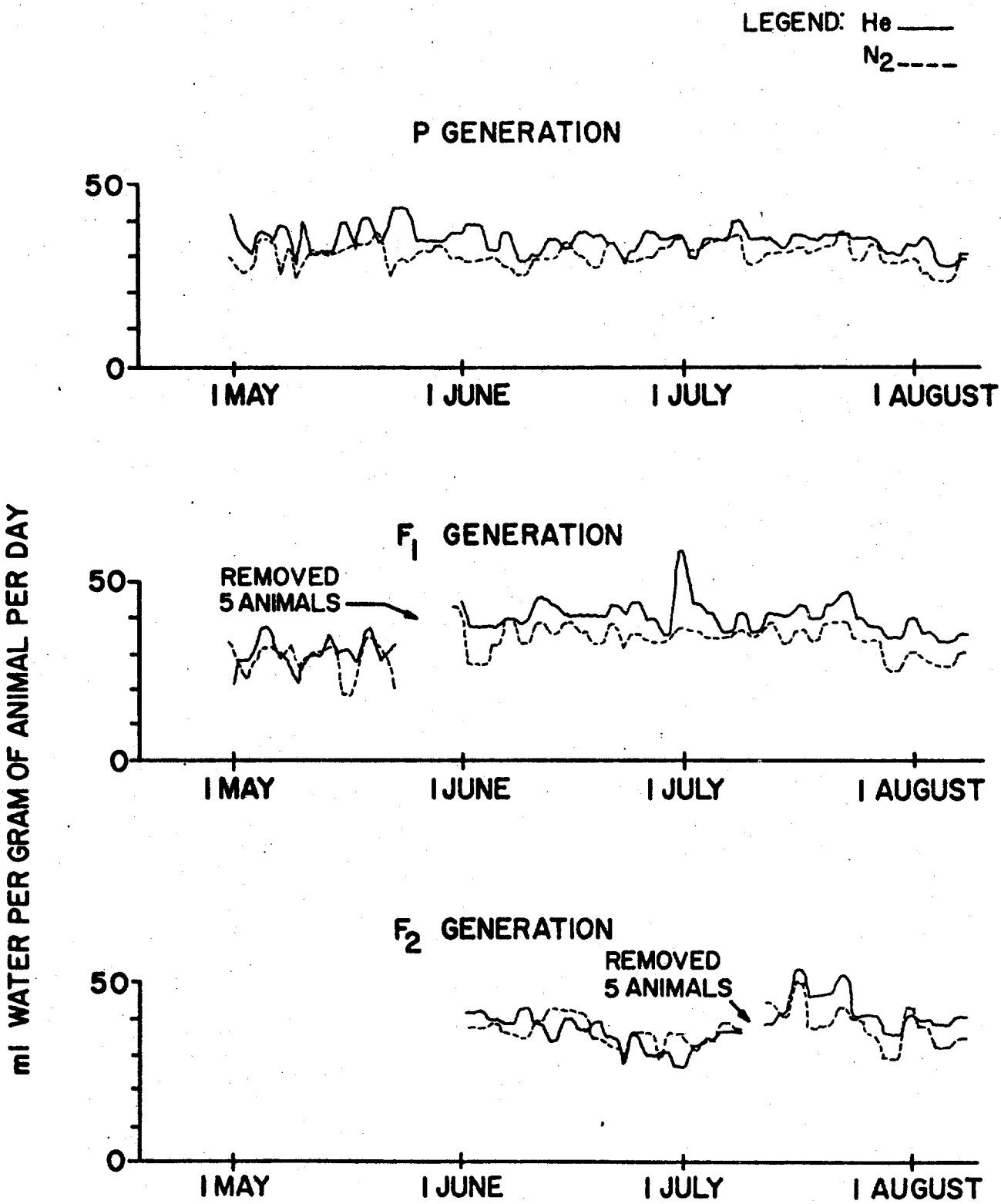


Figure 4. Water Consumption of Mice Chronically Exposed to Helium-Oxygen and Nitrogen-Oxygen at Ground Level Pressure.

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	100	37	11	Glock and McLean (1954)*
Dehydrogenase	100	22	12	This Study

Values are relative to highest activity tissue for each enzyme taken as 100 (see Dixon and Webb, 1964).

*Data by these authors are for rat tissues.

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)

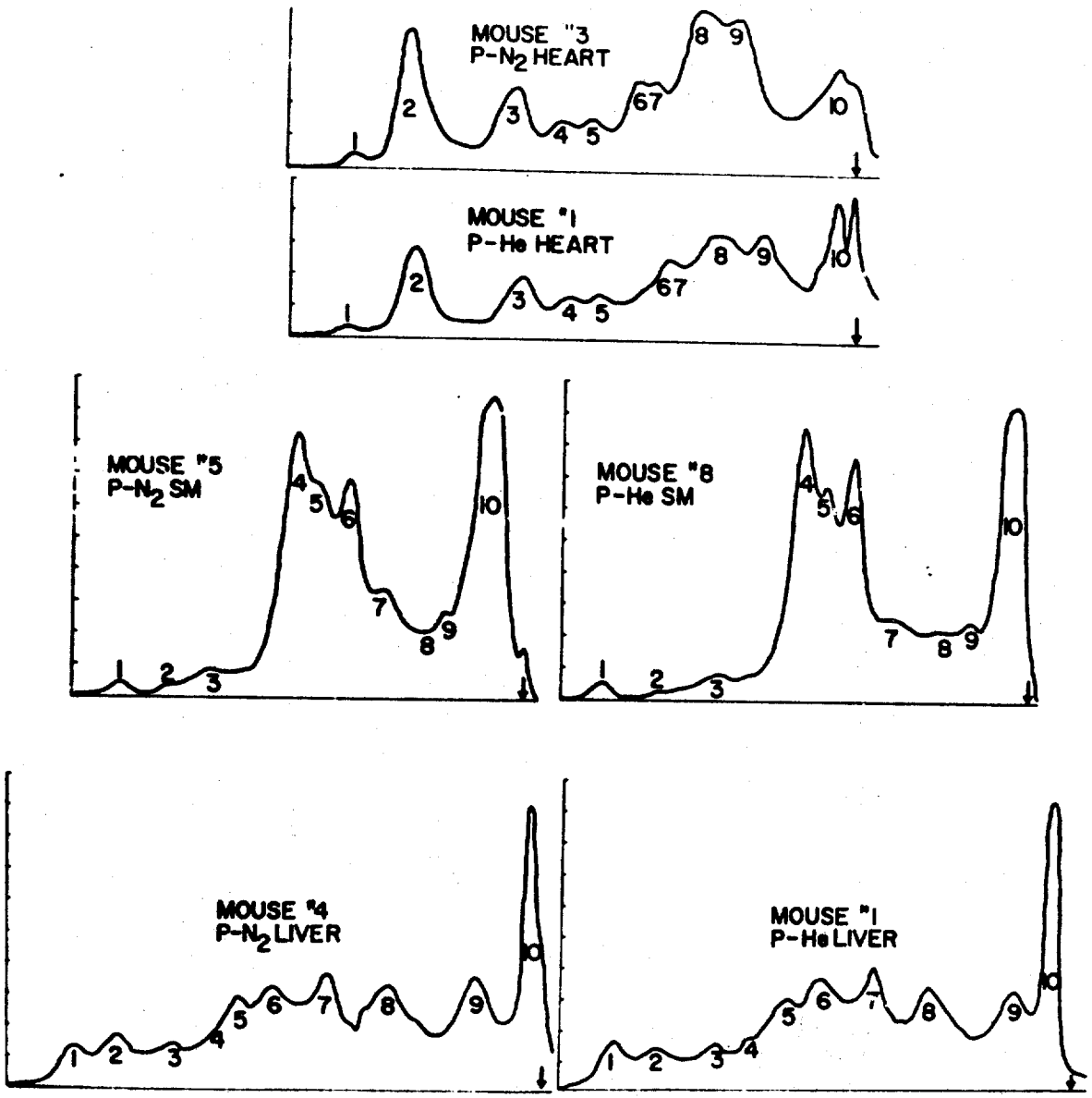


Figure 5. Representative Cellulose Acetate Strip Electrophoresis Patterns of Proteins of Mouse Tissue Extracts. Patterns are densitometer traces (arbitrary intensity scale) of actual strips. "P" refers to parent (initial) population; He and N_2 refer to animals from the helium-oxygen or nitrogen-oxygen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

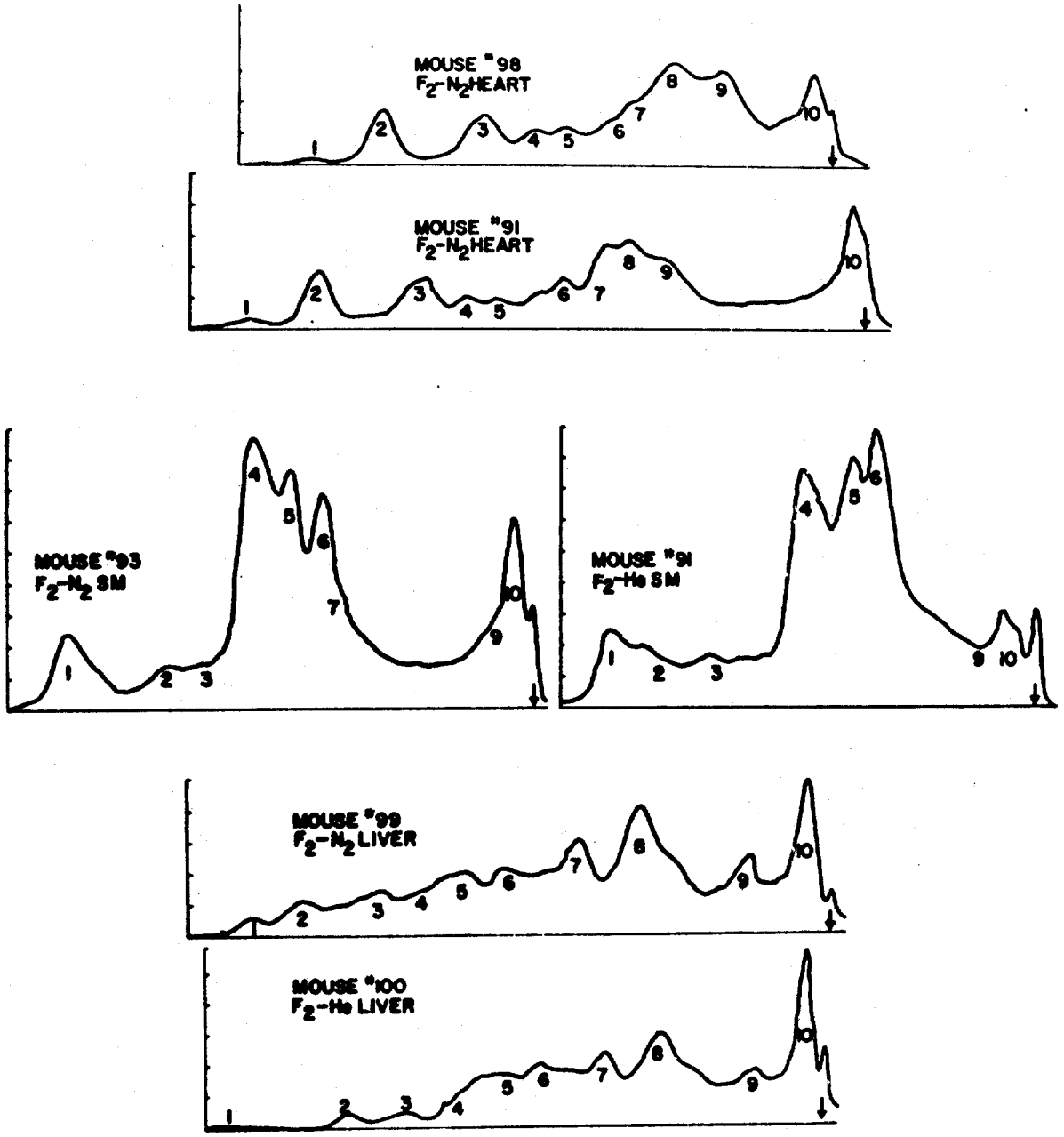


Figure 6. Representative cellulose acetate strip electrophoresis patterns of proteins of mouse tissue extracts. Symbols and conditions are as in Figure 5. F_2 refers to second generation animals born in the chamber environments.

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.

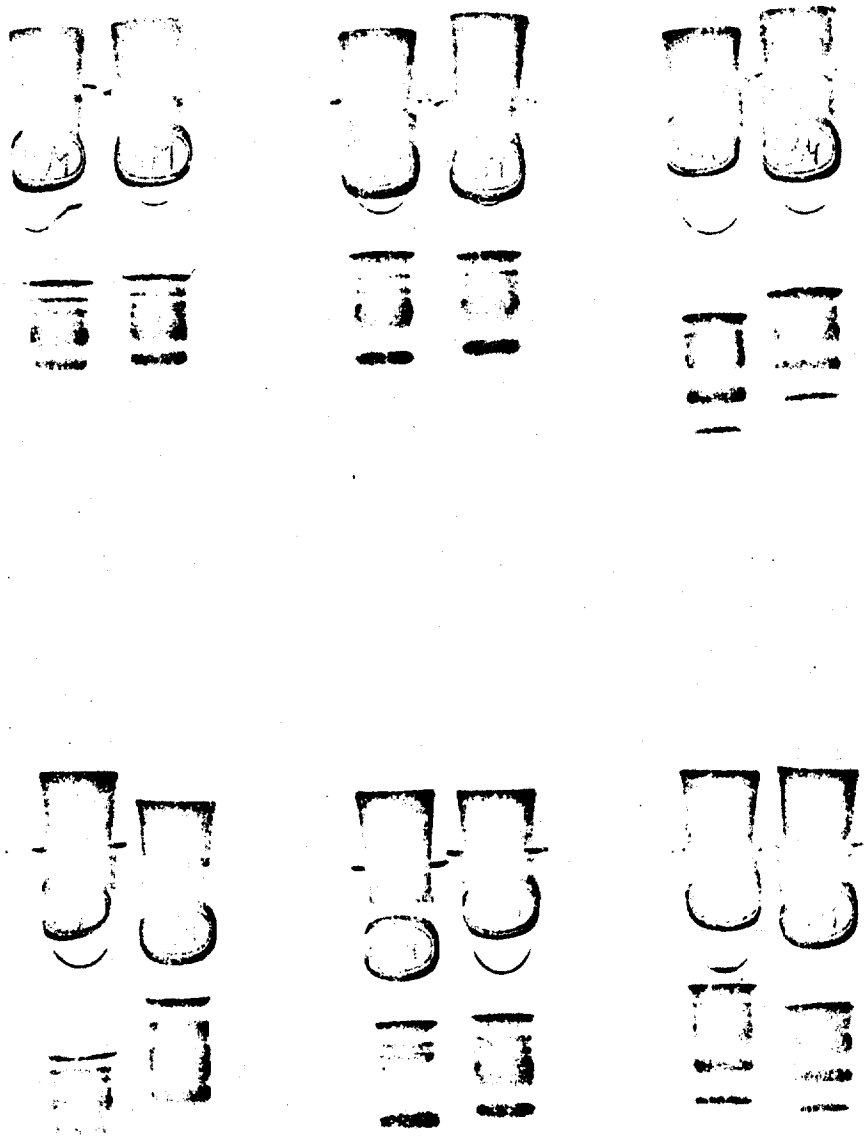


Figure 8. Photographs of Disc Electrophoresis Patterns of Proteins of Mouse Skeletal Muscle Extracts. Arrangement of gels as in Figure 7.

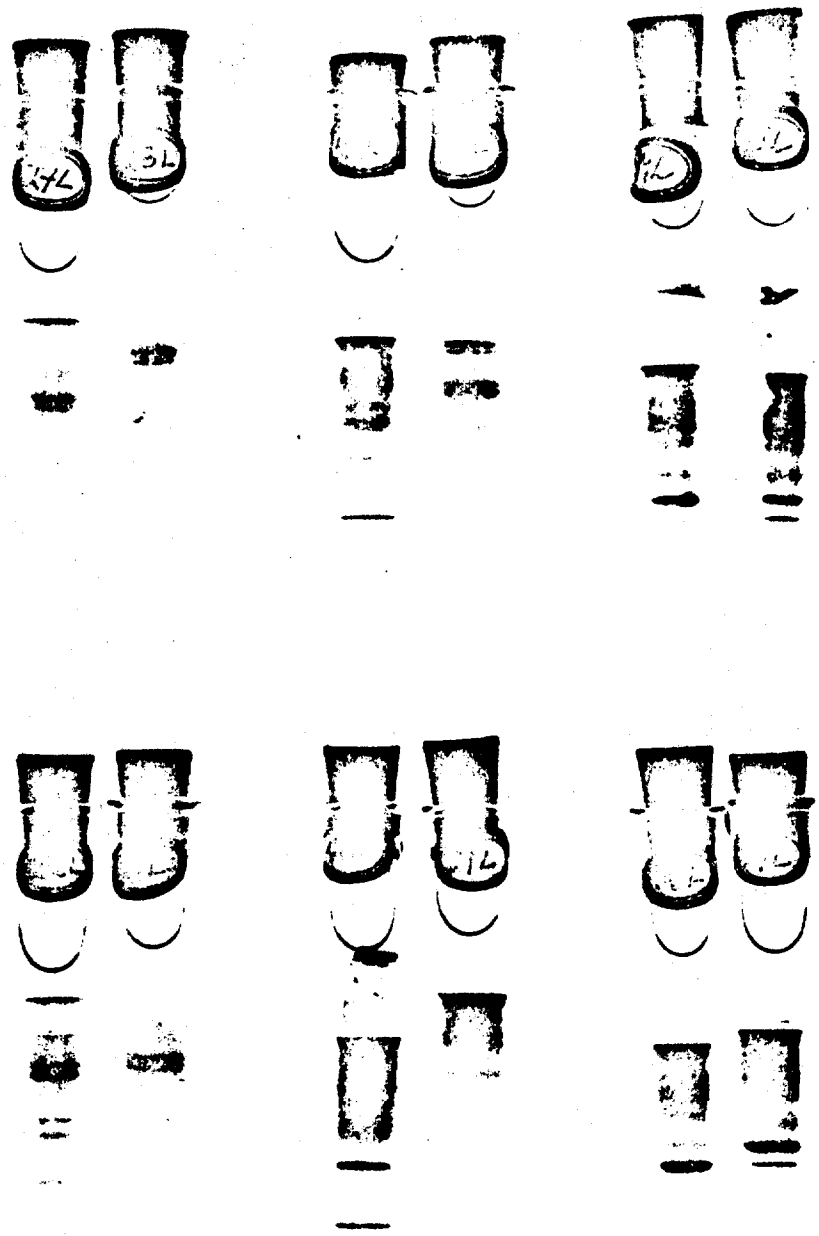


Figure 9. Photographs of Disc Electrophoresis Patterns of Proteins of Mouse Liver Extracts. Arrangement of gels as in Figure 7.

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; Cock and Leon, 1969; 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		Dry	
	He	N_2	He	N_2	He	N_2	He	N_2	He	N_2
P Generation										
$\bar{X}$	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).

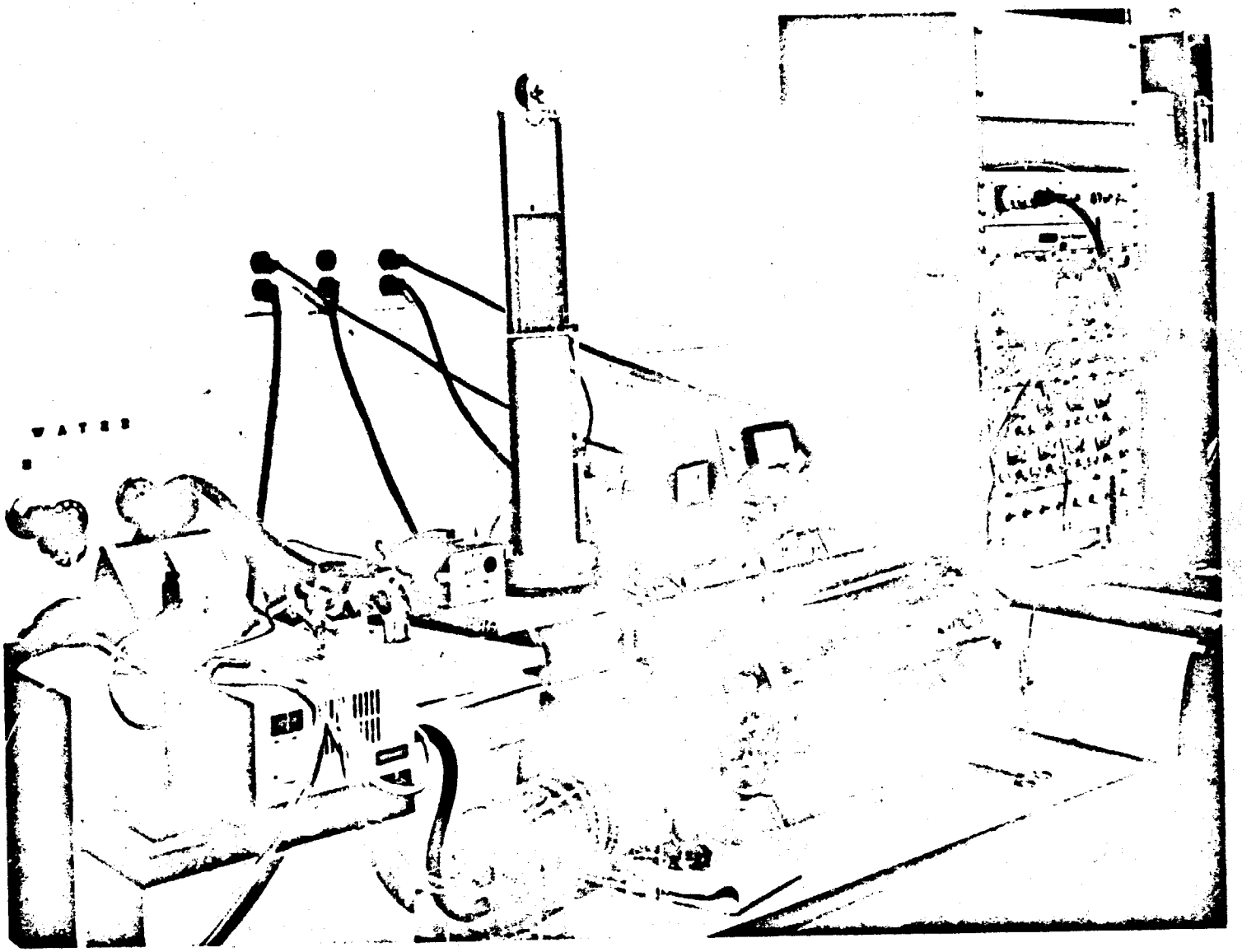


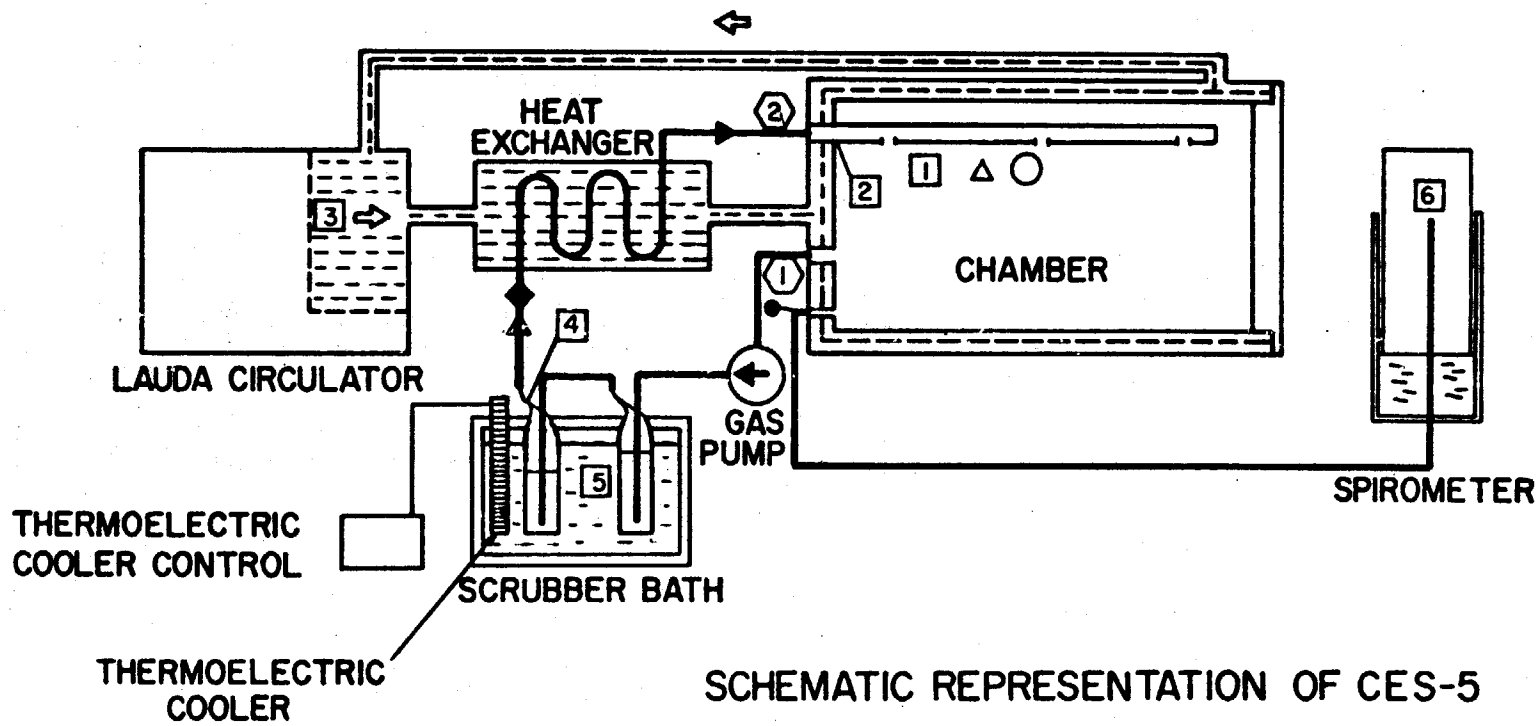
Figure 10. CES-5 in Operation. In the foreground, shown from left to right, are: the Lauda circulating water bath, gas heat exchanger, pressure transducer, animal chamber with two rats in restraining cages, and Offner recorder. In the background (from right to left) are the YSI cycling Telethermometer, 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-1 INFRARED CO_2 ANALYZER
- (2) RETURN FROM LB-1 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. × 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 macro-electrode. 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.

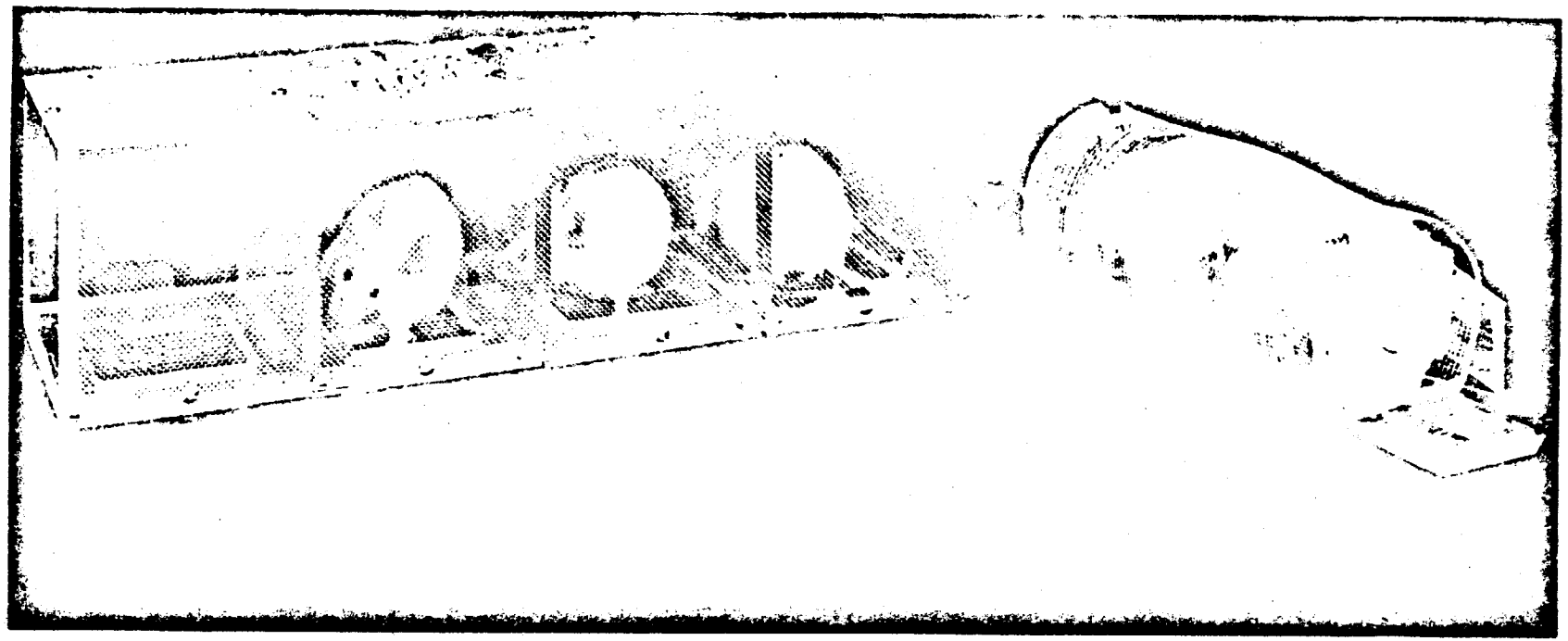


Figure 12. Restraining Devices for Mice and Rats for Oxygen Consumption Measurements in the CES-5.

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 Po_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	

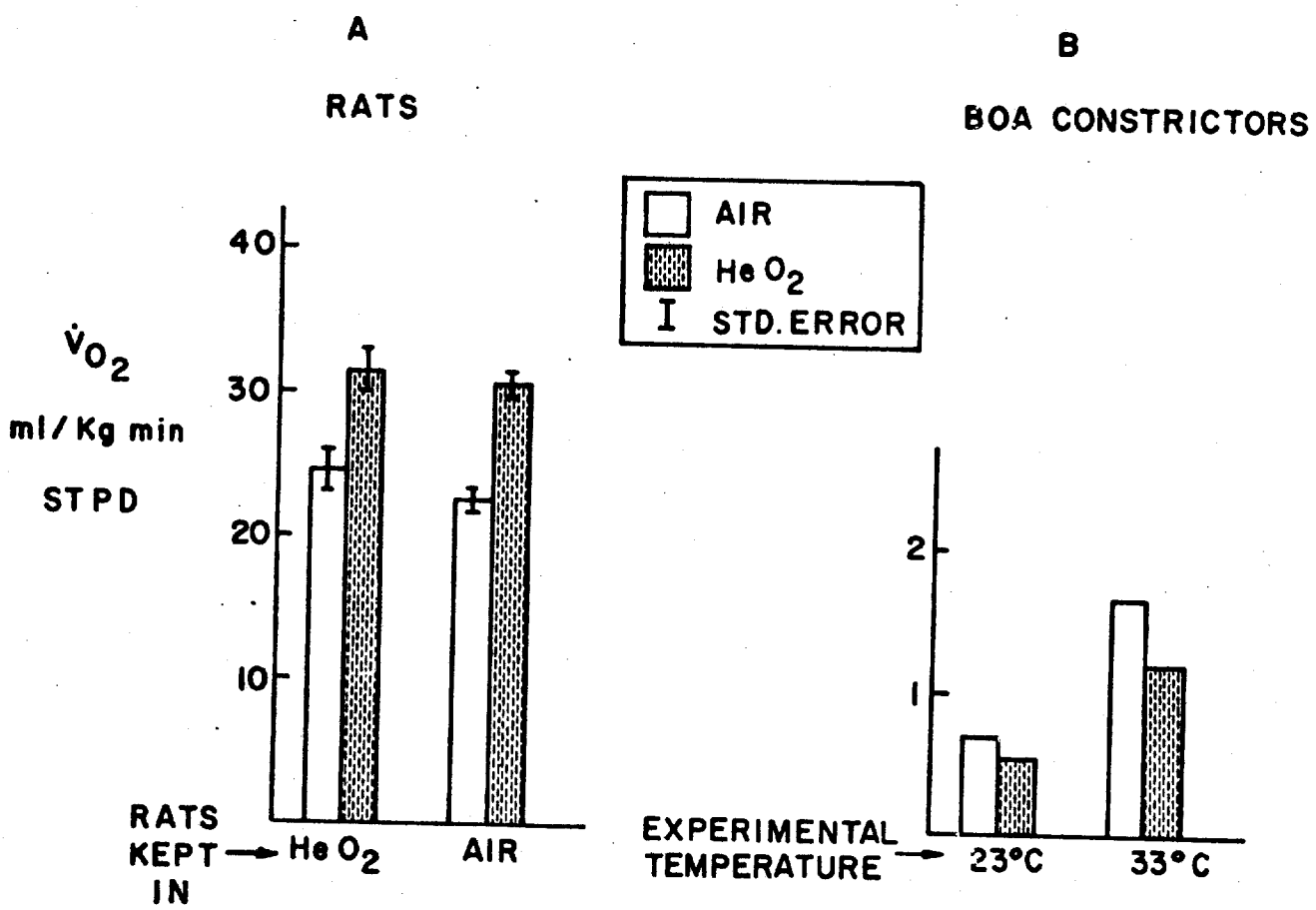


Figure 13. Oxygen consumption of Rats and Boa Constrictors. A: Comparison of response of air-raised (right) and helium-raised (left) rats with respect to their response to the HeO_2 atmosphere. B: Responses of boa constrictors to air and HeO_2 at two different temperatures. Boas were kept in air.

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	-

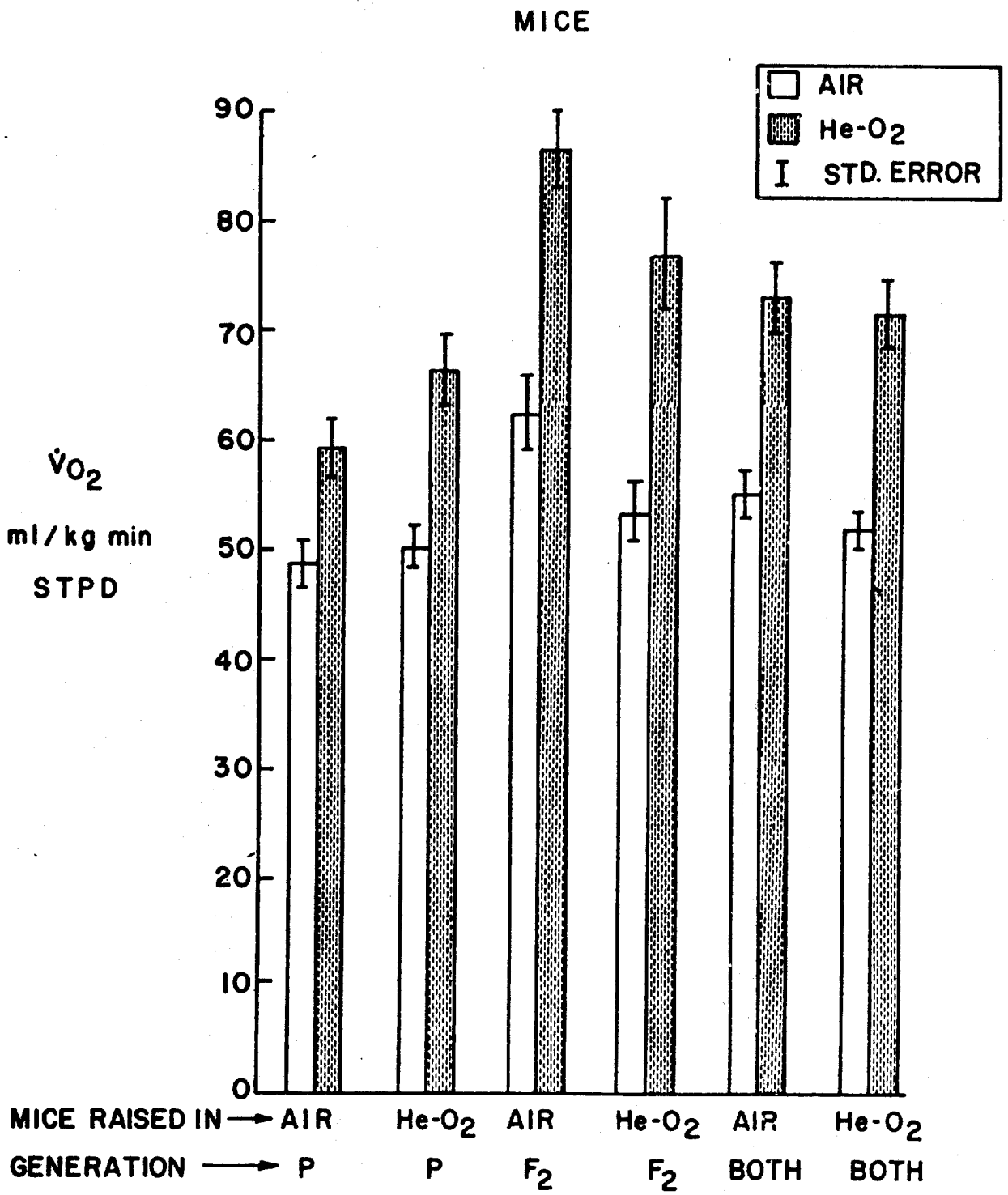


Figure 14. Oxygen consumption of mice. The open bars show the mean oxygen consumption of mice in air, and the shaded bars the consumption of the same mice in HeO_2. Order of presentation of the gases was mixed, and had no effect.

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

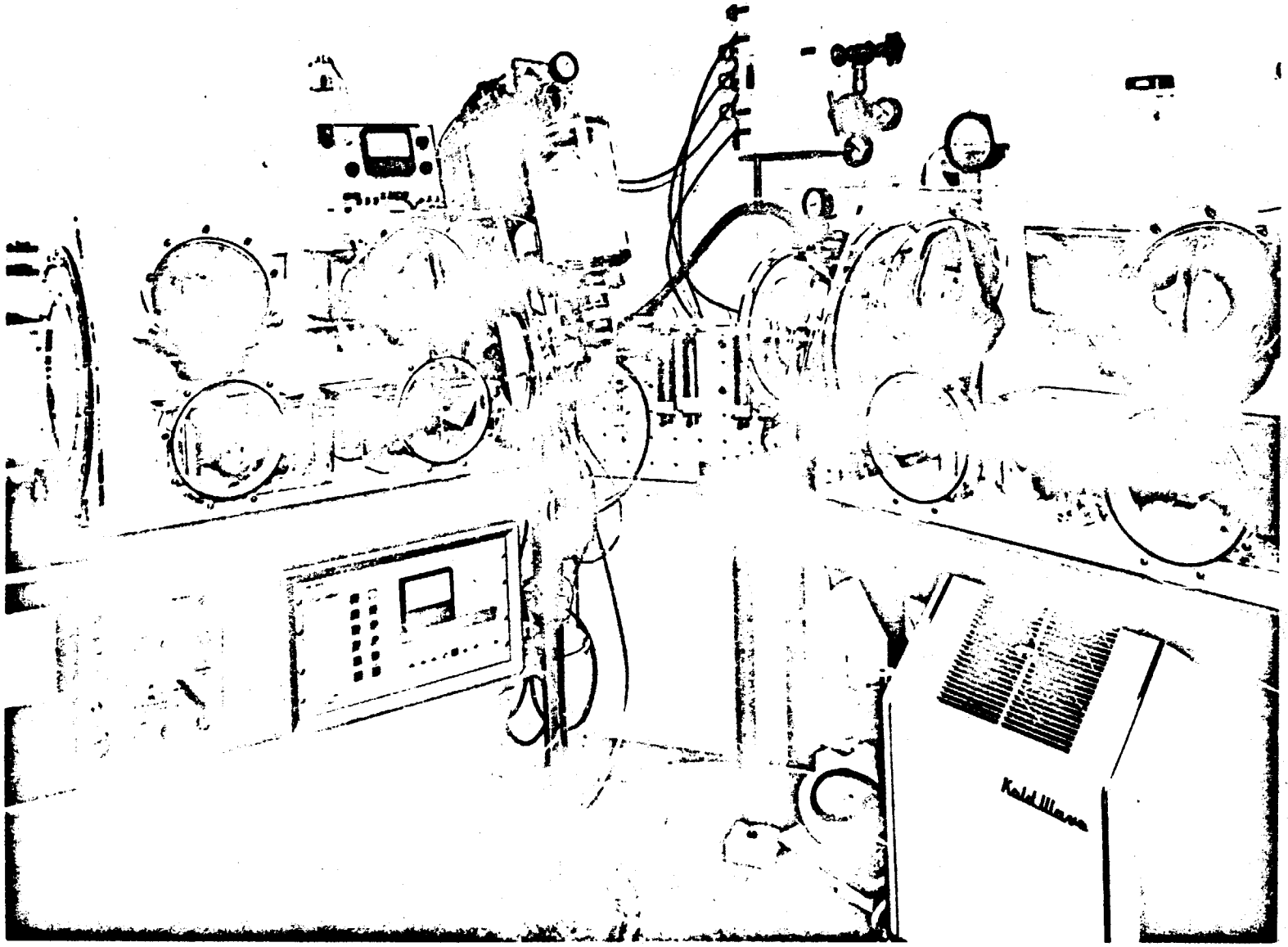


Figure 15. A General View of Controlled Environmental System 4.

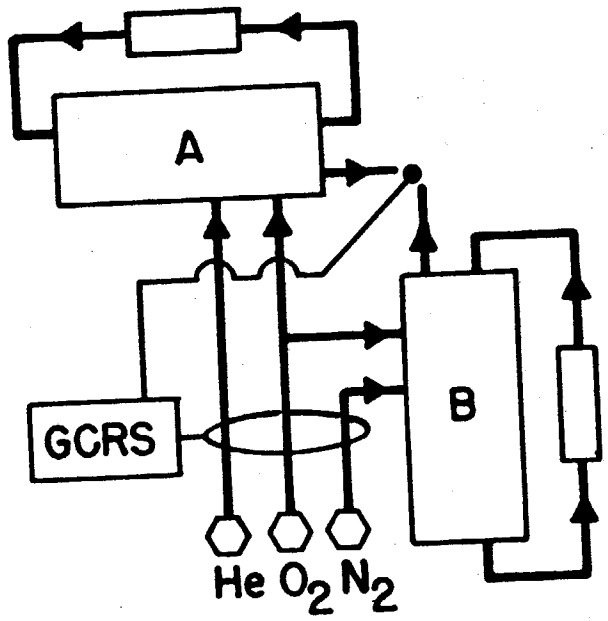


Figure 16. Overall Flow Diagram for the CES-4 System. The two chambers with their recirculating loops are shown, and the gas consumption regulating system as it relates to supply gas. The black dot represents the oxygen detector.

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.

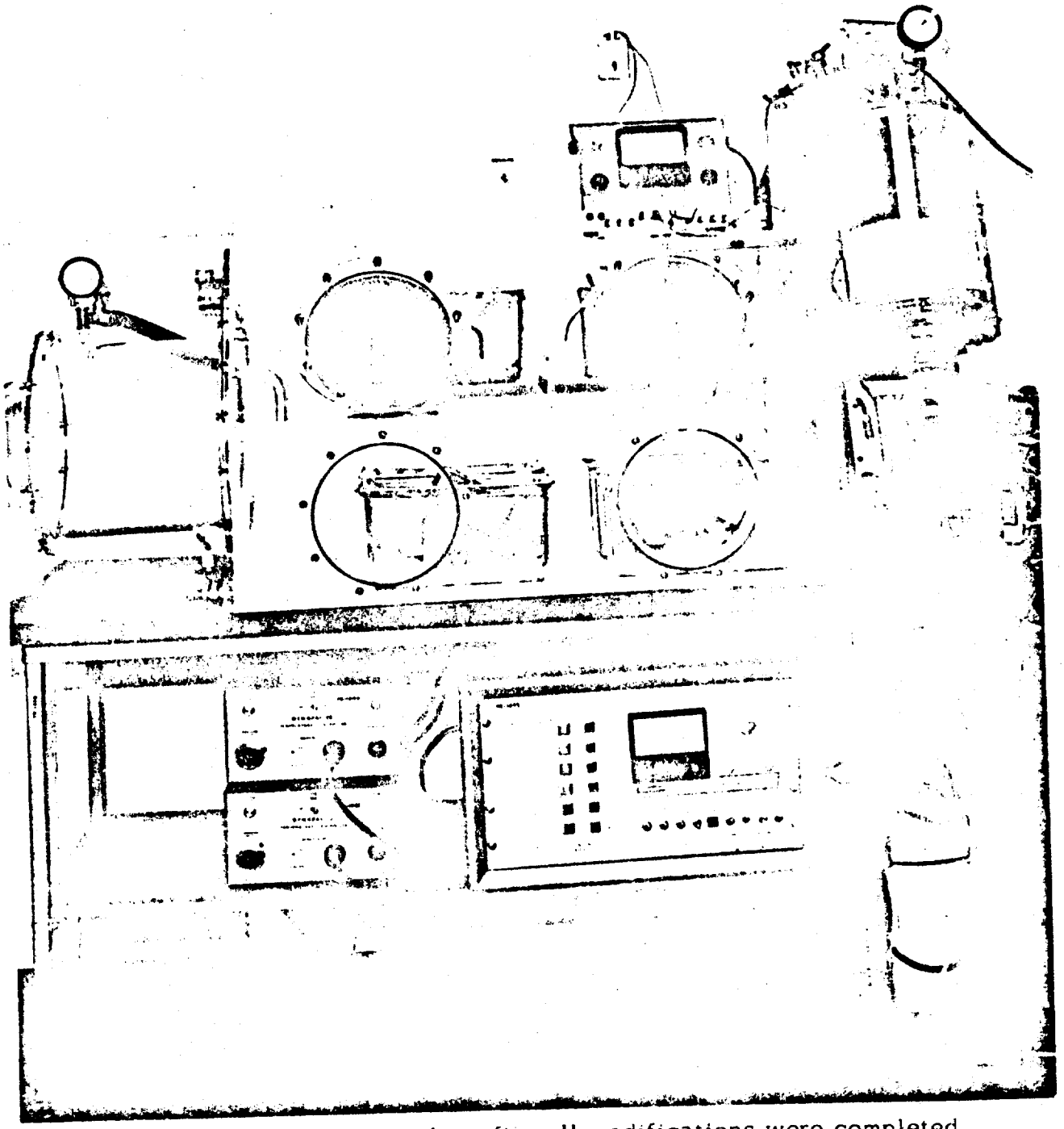


Figure 17. CES-4. Chamber after all modifications were completed.

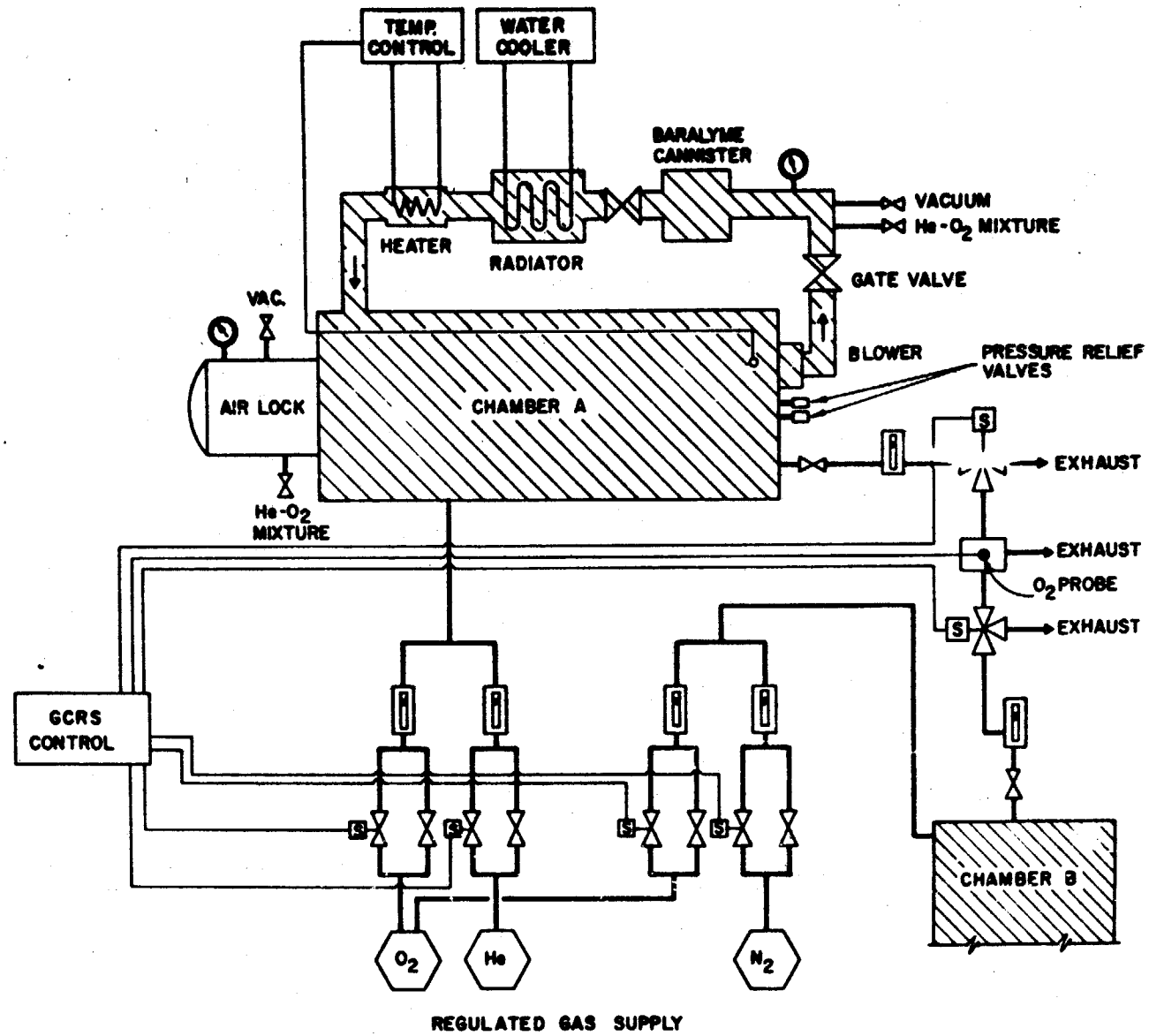


Figure 18. Detailed Schematic of the CES-4. Recirculation loop and accessories are identical for both chambers.

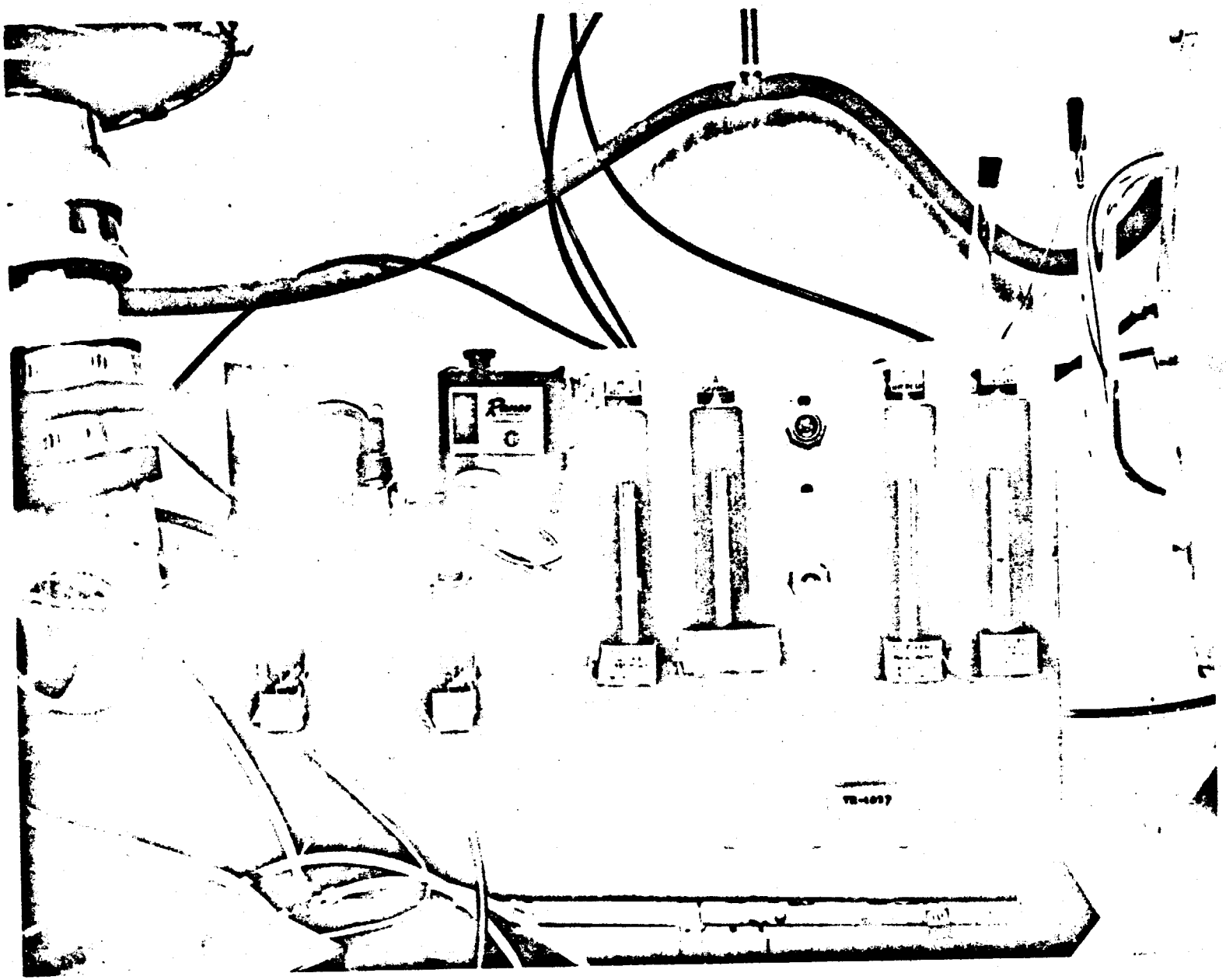


Figure 19. CES-4. Front view of Flow Meter panel.

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.

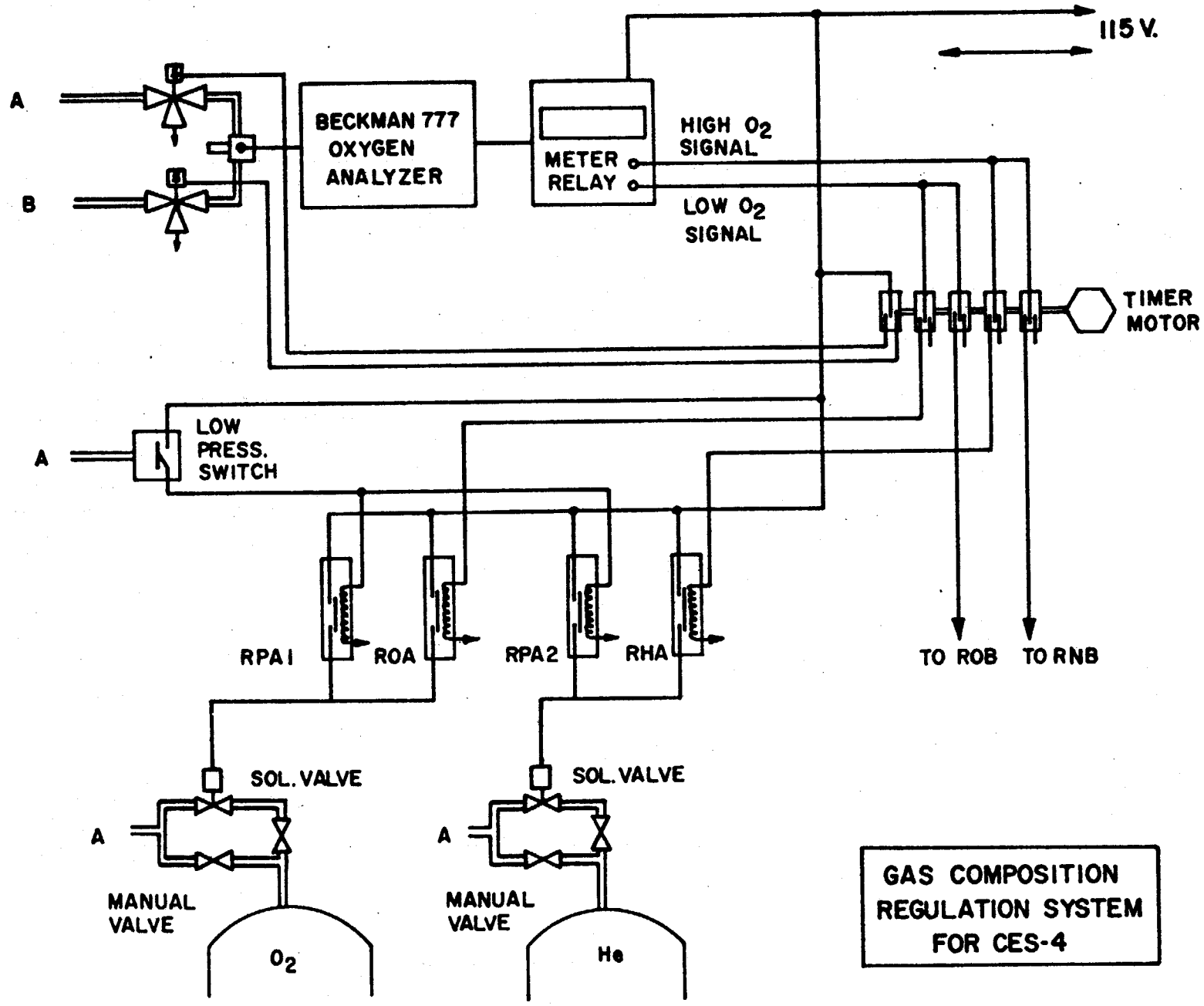
The timer (Industrial Timer Co.) has five circuits with programmable cams. The cams were programmed such that circuit one was used to alternate the exhaust of the chambers through the probe and the other circuits connected the output of the meter relay to the proper relay coil in synchrony with the oxygen 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.

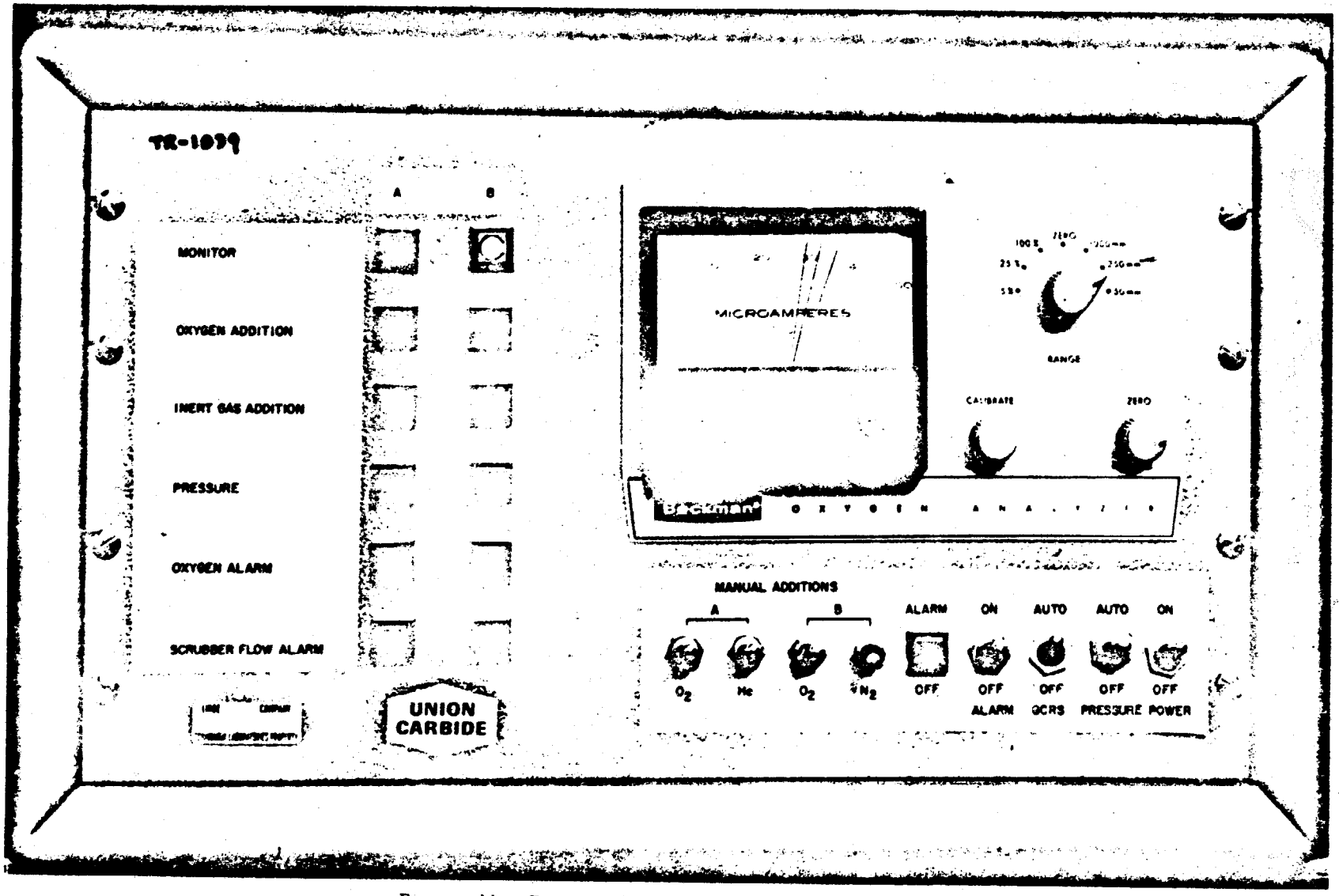


Figure 21. Front view of CES-4 Control Console.

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)

Compound	μ mole/gm tissue	
	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 $P < 0.05$.)

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Helium Effect on Cardiac Mitochondria of Mice

ISAAC K. HAWKINS
Medical College of Georgia, Augusta, Georgia 30902

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Twenty male A/Jax mice, 50 ± 5 days old at the onset, were exposed for 90 days at ambient pressure to the effect of substituting helium for nitrogen in respired air. Control animals were held under identical conditions, but their respiratory gas supply contained no helium. All animals were randomly divided into four equal groups. Each group was housed in a separate cage which was placed inside an individual environmental chamber. Electron micrographs of cardiac muscle from the helium treated animals showed varying degrees of diffuse disruption of the internal mitochondrial membranes and cristae. The mitochondrial disruptions were seen predominately in mitochondria adjacent to the transverse sarcotubular apparatus.

IN SEARCH OF METHODS of accommodating the metabolic requirements of astronauts and aquanauts we are confronted with the necessity of packaging part of the environment for transport. Barach and Eckman[1] suggested as early as 1934 that helium might be used to reduce the density of pulmonary gases. Dianov[4] in 1964 considered replacing nitrogen with helium in the artificial atmospheres of space vehicles but in 1964 Moody and Thomas[9] questioned the hypothesis of noble gas inertness, finding that helium group elements do form compounds with oxygen. Before man is subjected to basic alterations in his environment, these questions must be resolved.

In 1958 Ebert and Hornsay[5] found that helium had physiological properties that affected intracellular competition between oxygen and noble gases, and Boriskin[3] and Dianov[4] in 1962 pointed out that nitrogen removal affected oxygen metabolism. Bond[2] found an increased susceptibility to nitrogen narcosis in Sea Lab I individuals undergoing denitrogenation. Increasing the partial pressure of oxygen decreased its need by tissues exposed to noble gases. Schreiner[12] maintained that some physical property of noble gases exerted these effects upon tissues or whole organs, but this property is not related to their molecular weights. South and Cook,[13] and Rinfret and Doebbler[11] reported that helium affects the cyanide-cytochrome system and the citric acid cycle. No *in vivo* examination has been made of the ultrastructural effects of helium on cardiac muscle. This study examines the effect upon cardiac mitochondria of substituting helium for nitrogen in the respiratory environment.

MATERIALS AND METHODS

Twenty male, A/Jax mice, 50 ±5 days old at the onset, were used in this study. The animals, weighed and marked, were randomly divided into four groups of five animals in each group. Two of the groups served as control animals. Each group was housed in a disposable, rigid, plastic, wire-topped cage, 5 × 11.5 × 7.5 inches, and each animal cage was placed inside a flexible, plastic, environmental chamber. The plastic animal cages were modified with effluent ports along each side one inch from the bottom. Wire cage tops supported food and water containers. Dried sugar-beet pulp was used for litter to a depth of 1.5 inches. Care of the animals was performed inside the environmental chambers, the animals not being removed from the chamber until termination of the experiment. The experiment was conducted in an isolated room.

Test atmospheres were obtained from compressed bottles of 80% helium and 20% oxygen, USP. Atmospheres for the control animals were taken from compressed bottles of 70% nitrogen and 21% oxygen, USP. Two flexible, plastic chambers were attached to each of the bottled gases distal to intervening reduction valves. The chambers were constructed of large, heavy-duty, clear, polyethylene plastic bags for the admittance of light, animal care and convenience in viewing. The flexibility of the chambers created a nonrigid system which, under barometric differentials, could result in unequal gas flow to each group of animals. To regulate gas flow to each chamber to a constant 0.5 scfh, 500 ml. erlenmeyer flasks filled with 400 ml. of glycerine, USP, were placed between each chamber and its gas supply. Moisture and mold growth inside each chamber and cage were controlled with indicating Drierite. Carbon dioxide was absorbed with indicator soda lime, USP. Lighting was regulated to alternating 12-hour periods of light and dark.

At termination of the experiment all animals were weighed, anesthetized by intraperitoneal injections of undiluted Somnopentyl (0.001 cc/gm of body weight) and the tissues removed while the animals were still living. Tissue taken from the tip of the left ventricle was immediately minced in a drop of chilled gluteraldehyde, and then immersed in chilled 6.5% gluteraldehyde, buffered to pH 7.4 with 0.1M sodium cacodylate, prior to embedding in Vestopal-W.[10]

Tissues were sectioned on an LKB ultramicrotome, Type 8801A, with a control unit Type 8802A. Sections made at 600 Å thickness were mounted on 200 mesh, 3.0 mm, copper grids prepared with formvar and carbon, and stained with osmium tetroxide, uranyl acetate and lead citrate.[8] The grids were examined with RCA EMU-3 electron microscope.

RESULTS

Electron micrographs of cardiac tissue from control animals showed no change in the mitochondria (Figure 1). Cardiac muscle from the control and helium treated animals showed no change due to hypoxia as reported by Heggtveit[7] (Figures 1 and 2). Random changes occurred in mitochondria (Figure 2). The most severe alteration in the mitochondria was usually located near the transverse sarcotublar apparatus (Figure 2). There was no apparent change in the number of mitochondria, but the number of cristae in the affected mitochondria decreased. In some of the affected mitochondria the outer and inner mitochondrial membranes appeared to be ruptured.

DISCUSSION

The substitution of helium for nitrogen in the respiratory gases involves consideration of the low molecular weight, high thermal conductivity, low solubility and high diffusion rate of the helium group gases. In 1961 Rinfret and Doebbler[11] considered the possible metabolic effects of noble gases. With the exception of radioactive radon, these gases exhibited physiological properties which relate to their periodic classification. They also established, *in vitro*, that helium inhibits phosphorylation reactions, interferes with cyanide-cytochrome oxidase bonds and increases oxygen consumption and carbon dioxide production. South and Cook[13] found that helium increased the rate of glucose oxidation, accelerates glycolysis anaerobically between the reactions involving the enzymes aldolase and enolase, inhibiting glycolysis prior to the formation of hexose diphosphate, may involve the tricarboxylic acid cycle and may have some effect on the cyanide-cytochrome bond system. Rinfret and Doebbler[11] explained these effects on the basis of membrane adsorption of helium, and many of these reactions involve mitochondria. Physical alteration of the membrane systems of cells as seen in this study might influence any or all of these metabolic processes.

Helium substitution for nitrogen could involve any of the interrelated metabolic systems, presenting the possibility of many biophysiological effects.[6] Helium increases oxygen needed as it reduces the density of respired gases, and it exerts a narcotizing effect hyperbarically. Schreiner[12] reported that metabolic effects are inconsistant with the molecular weights, and that helium could affect several sites in the metabolic cycle(s), not being limited to any particular metabolic process. The biophysiological action of helium may vary from surface membrane effects to chemical union.

As designed in this study the exposure of animals to helium necessitated a removal of nitrogen. The metabolic effects of nitrogen removal are not fully understood. Although the *in vitro* results reported by Rinfret

Fig. 1. Control animals. The transverse sarcotubular apparatus (ts), mitochondria (m), and mitochondrial cristae appear unaltered. x40,000

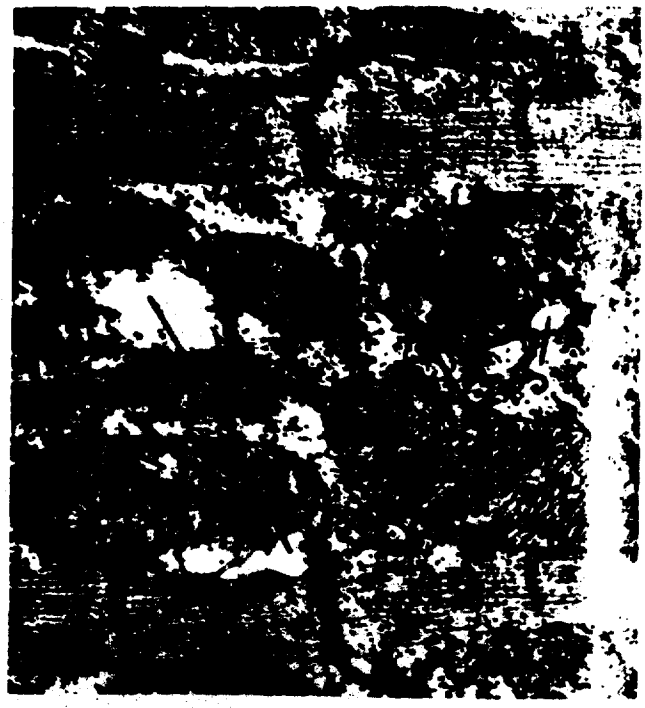

Fig. 2. Test animals. Severe bleb (b) formation in the mitochondria appear adjacent to the transverse sarcotubular apparatus (ts). Smaller interruptions (c) can be seen in the cristae of the affected mitochondria. x42,000

and Doebbler[11] on homogenates and slices could have occurred because of rearranged membrane surfaces, it is logical to assume that effects seen in this study were produced by helium with the role of nitrogen removal being negligible.

The lesions seen in the cardiac muscle represent ultrastructural evidence that muscle metabolism likely was affected. Young test animals demonstrated net weight gain(s) over the 90-day treatment period, indicating that the animals were adapting to the new environment. This adaptation indicated metabolic compensation in a helium atmosphere free of nitrogen.

Rinfret and Doebbler[11] wrote, "It is reasonable to conclude that marked difference in susceptibility to gases of the helium group may exist between the tissues of an organism as well as the physiological systems functioning within them."

SUMMARY

Mitochondrial cristae appeared to be altered randomly and diffusely in some mitochondria adjacent to the transverse sarcotubular system. Some of the mitochondrial membranes appeared to be ruptured or protruding into the transverse sarcotubular cisternae. In assessing the mitochondrial changes in this experiment, two points must be considered: (1) the bio-physiological effects of the rare gas elements are poorly understood, and (2) the effect of removing nitrogen from an animal which has evolved in a nitrogen-rich environment is relatively unexplored.

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Helium Effect On Skeletal Muscle Of Mice

ISAAC K. HAWKINS
Medical College of Georgia, Augusta, Georgia 30902

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Twenty male A/Jax mice, 50 ± 5 days old at the onset, were exposed for 90 days at ambient pressure to the effect of substituting helium for nitrogen in respired air. Control animals were held under identical conditions, but their respiratory gas supply contained no helium. All animals were randomly divided into four equal groups. Each group was housed in a separate cage which was placed inside an individual environmental chamber. Photomicrographs of skeletal muscle from the helium-treated animals showed diffuse myopathic lesions of some of the skeletal muscle fibers. Additional indications of muscle tissue degredation were present.

IN 1958 EBERT AND HORNSEY[6] found that helium had physiological properties that affected intracellular competition between oxygen and noble gases; and Boriskin[3] pointed out that nitrogen removal affected oxygen metabolism. In 1964, Moody and Thomas[11] questioned the hypothesis of noble gas inertness, finding that helium group elements do form compounds with oxygen. Increasing the partial pressure of oxygen decreased oxygen need by tissues exposed to noble gases. Schreiner[13] maintained that some physical property of the noble gases affected tissues or whole organs, but this property is not related to their molecular weights. South and Cook[15], and Rinfret and Doebbler[12] reported that helium affects the cyanide-cytochrome system and the citric acid cycle. No *in vivo* examination has been made of the microscopic effects of helium on skeletal muscle of mice. This study examines the effect upon the morphology of skeletal muscle fibers of substituting helium for nitrogen in the respiratory environment.

MATERIALS AND METHODS

Twenty male, A/Jax mice, 50 ± 5 days old at the onset were used in this study. Test atmospheres were obtained from bottles of compressed 80% helium and 20% oxygen, USP. Atmospheres for the control animals were taken from bottles of compressed 79% nitrogen and 21% oxygen, USP. The animals were maintained as previously reported by Hawkins.[8]

At termination of the experiment all animals were anesthetized by intraperitoneal injections of undiluted Somnopentyl (0.001 cc/gm of body weight), and the left femur and accompanying musculature removed while the animals were still living. The femurs were separated

Fig. 1. This cross-section of skeletal muscle from the control animals shows some connective tissue elements but no centrally located nuclei. Skeletal muscle nuclei, (s), and fibroblasts, (f), are indicated. Magnification ×540.

above the hip joint and at the stifle joint to prevent damage to the fragile bone or disruption of the delicate musculature. The tissue specimens were immediately immersed in chilled Susa's fixative.[9] After 24 hrs the tissues were removed from the fixative and washed in cold, running, tap water for 3 hrs, then placed in 70% ethyl alcohol as an initial step in dehydration preparatory to embedding in Paraplast, utilizing the standard paraffin method. Tissue blocks were allowed to stand a minimum of 24 hrs before sectioning.

Spaced-serial sections were taken at 7.0 microns on a Leitz rotary microtome, and the sections mounted on standard 1 x 3 inch glass slides in preparation for staining. The sections were post-treated for mercuric chloride for 2 min with 0.5% iodine in 80% ethyl alcohol followed by 5 min in 0.5% aqueous sodium thiosulfate in order to remove the iodine.[9] The removal of mercuric chloride and iodine was done in the hydration procedure between 95% and 70% ethyl alcohol just prior to staining with Harris alum hematoxylin followed by a counterstain in 1.0% alcoholic eosin Y solution for 3 min. The slides were then dehydrated and coverslipped.

RESULTS

Photomicrographs of skeletal muscle tissue from control animals showed no indication of muscle degredation (Fig. 1). In the helium-treated animals diffuse myopathic lesions were found. The severity of the myopathy varied among the animals exposed to the helium. Individual muscle fasciculi varied in the response to helium. In some

Fig. 2. Variations in the tissue response are present in this cross-section of skeletal muscle from the helium-treated animals. Isolated fibers, (a), and contiguous groups of several fibers, (b), show a gradation in the myopathic alterations. Some muscle nuclei, (s), appear to be migrating from the usual hypolemmal position. The affected fibers, (a and b), show a rounded morphology in transverse section, differing from the polyhedral cross-sectional configuration seen in Fig. 1. A general increase in the epimysial connective tissue is also present. An artifact, (x), is present in lower portion of the figure. Magnification ×270

Fig. 3. This montage of skeletal muscle from the helium-treated animals show muscle tissue in cross and tangential sections. In the tangential section the fusiform remnants of muscle fibers are seen beaded along the length of the muscle cell. At least three muscle fibers, (1,2,3), have been severely affected. An increase in the connective tissue elements is present. The basophilic masses inside the beads appear to be remains of the sarcoplasm, (r). Magnification ×150.

sections (Fig. 5), myopathic lesions are adjacent to skeletal muscle tissue which appears unaffected. The basophilia of the affected fibers varied from slight to intense, with the affected fibers grouped in isolated fibers or in contiguous groups of several fibers (Fig. 2). The most severe lesions were fusiform, the fusiform clumps being beaded along the length of the fiber (Fig. 3).

In some of the skeletal muscle fibers there was evidence of what may have been attempts of regeneration. These were (1) rounding of the cross-sectional fibers (Fig. 2), (2) migration of hypolemmal nuclei from a mature, peripheral location to an embryonic, central lo-

Fig. 4. In this cross-section of skeletal muscle from the animals exposed to helium, nuclei, (n) are seen in the central portion of some of the muscle fibers. The round, circular fiber, (O), resembles an embryonic sarcotube. The centrally located nuclei, (n), appear pyknotic. An increase in the extracellular, connective tissue elements is present. Magnification ×5[illegible]0.

cation (Fig. 4), and (3) formation of round, circular fibers resembling embryonic sarcotubes (Fig. 4). Indications of muscle tissue degredation were present. These indications were a progressive shift in the staining characteristic of the myopathic myoglobin toward the intense basophilia observed in the more severe lesions (Fig. 3), and a generalized, diffuse increase in endomyseal and perimyseal connective tissue (Figs. 4 and 5). No acute inflammatory response was found. In the tangential sections (Fig. 3), and cross-sections of the fibers most severely affected (Fig. 5), many cellular elements were present, i.e. lymphocytes, fibroblasts, muscle nuclei and remnants of endomyseal and perimyseal connective tissue (Fig. 5). In the myopathic fibers seen in Figs. 3 and 5, endomyseal and perimyseal connective tissue may contain membrane vestiges of the muscle fibers. The other tex components appear to have parted away from the sarcolemma with the sarcoplasm leaving the muscle cell membrane adjacent to the endomysium.

DISCUSSION

In 1961, Rinfrêt and Doebbler[12] considered the possible metabolic effects of noble gases taking into account their molecular weights, thermal conductivity, low solubility, and diffusion rates. Other than radioactive radon, these elements exhibited physiological properites which relate to their periodic classification. They also established, *in vitro*, that helium: inhibits phosphorylation reactions; interferes with cyanide-cytochrome oxidase bonds; and increases oxygen consumption and carbon

Fig. 5. This cross-section of skeletal muscle from helium-treated animals shows an increase on the cellular elements. The number of skeletal muscle nuclei, (s), appear to have increased. Some of these nuclei are migrating toward mature, hypolemmal positions, or toward embryonic, central locations. Remnants of endomyseal and perimyseal connective tissue, (c), are present. An unaffected fasicle of muscle tissue is indicated, (a). Magnification ×540.

dioxide production. South and Cook found that helium increased the rate of glucose oxidation; accelerates glycolysis anaerobically between the reactions involving the enzymes aldolase and enolase, inhibiting glycolysis prior to the formation of hexose diphosphate; may involve the tricarboxylic acid cycle; and may have some effect on the cyanide-cytochrome bond system. Helium substitution for nitrogen, involving interrelated metabolic systems, could possibly present many biophysiological effects.[14]

Schreiner reported the metabolic effects are inconsistant with the molecular weights, and that helium could affect several sites in the metabolic cycle(s), not being limited to any particular metabolic process. The biophysiological action of helium may vary from surface membrane effects to chemical union. In this study the exposure of animals to helium necessitated a removal of nitrogen. The metabolic effects of nitrogen removal are not fully understood[3,4]. Although the *in vitro* results reported by Rinfret and Doebbler on homogenates and slices could have occurred because of rearranged membrane surfaces, it is logical to assume that effects seen in this study were produced by helium. South and Cook[15] stated, ". . . since the respiratory quotient remained the same as in air, the same intracellular substance is being metabolized in helium as in air."

The myopathic lesions seen in the skeletal muscle fibers are evidence that muscle metabolism likely was affected. Interference of cell metabolism by helium probably occurred at the organelle level.[5] The intense, basophilic myopathies of the degenerated muscle fibers most likely are associated with ultrastructural changes observed in helium-exposed animals.[5] The experimental animals demonstrated one of the gross symptoms attributed to muscular dystrophy when suspended by their tails in midair. This was a clasping of the hind limbs.[7] In myopathic disturbances various enzyme systems are suspect, such as succinic dehydrogenase,[1] glycine transamidinase,[5] and the alph-, beta-, and gamma-tocopherols.[10] Since helium is known to influence the citric acid cycle, the cyanide-cytochrome oxidase enzyme system, and inhibit and accelerate various glycolytic processes, and since glucose metabolism is affected in the dystrophic mouse, then the conclusion that helium may in some way effect a dystrophic-like myopathy in murine skeletal muscle must be considered. The primary etiology of the clinical and histologic symptoms of muscular dystrophy are unknown, but the biochemical enzyme systems affected by helium and muscular dystrophy are similar. These similarities are (1) mitochondria appear swollen and less dense, and they are progressively lobulated and disintegrated,[2] indicating a disturbed oxidative enzyme activity, (2) membrane permeability is altered, (3) glycolysis is affected with a 60% higher efflux of aldolase and a concommitant high serum concentration of aldolase, and (4) ringed fibers are evident histologically with basophilia appearing in the cytoplasm of the degenerating or regenerating fibers.[8]

It is reasonable to conclude that marked differences in susceptibility to gases of the helium group may exist between the tissues of an organism as well as the physiological systems functioning within them.[13]

SUMMARY

In assessing the changes found in the skeletal muscle in this study, two points must be considered: (1) the biophysiological effects of the rare gas elements are poorly understood, and (2) the effect of removing nitrogen from an animal which has evolved in a nitrogen-rich environment is relatively unexplored. Whether or not the myopathies are a type of muscular dystrophy will require further investigation.

The lesions appeared to be ovoid remnants, of the sarcoplasm and membrane systems of the muscle fibers, which were being isolated by connective tissue elements. Some evidence of random regeneration was present. The destruction of the skeletal muscle tissue required time to manifest the chronic microscopic characteristics observed, and the gross signs of muscular myopathies were subliminal.

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ANÆSTHETIC TOXICITY

By V. E. Henderson, M.B.

Department of Pharmacology, University of Toronto

THE subject of toxicity of our common general anæsthetics has been so much discussed that it is very difficult to contribute anything new in the way of facts. There is, however, a point of view which I think is too frequently overlooked in discussions regarding anæsthetic toxicity. Of this there may be two types. Toxic action and death may be due: (1) to overdosage or improper administration; or (2) may occur as a result of the essential toxicity of the anæsthetic, although the ordinary bounds of dosage and concentration may not have been exceeded. These statements will require further elaboration. All our anæsthetics are of course toxic, that is, they produce symptoms arising from pathological or pharmacological changes, if given in overdoses. This is true of practically all our drugs. The site of the dangerous action, however, differs greatly. For example death from chloroform may be due to the heart, whereas with ether this is exceedingly rarely the case. Butylene anæsthesia induced slowly, produces a hypertonus resembling cerebrate rigidity to a much greater extent than propylene, and indeed, in my experience, more readily than any other of our anæsthetics, though this may occur in various animals even with ether and chloroform. The action of butylene on the frontal lobes, which are concerned with tonus mechanisms, seems to be much greater than that of the other anæsthetics. This fact alone would detract from butylene being a useful anæsthetic, since the hypertonus often persists to a deep stage of anæsthesia. Butylene has another effect, namely, it tends in anæsthetic doses to cause a very marked fall of blood pressure, doubtless due to a depression of the vasomotor centre. While, therefore, we may talk of the toxicity of an anæsthetic in a general sense, if we wish to speak intelligently we must be precise in our comparisons and indicate the site in the organism where the deleterious effect of the anæsthetic is produced. In regard to toxicity occurring from overdosage or improper use I shall have relatively little to say, but it is obvious that the anæsthetic of choice, if it is to be employed by the many and not merely by the expert, should show as wide a margin as possible between the dosage in which it exerts deleterious actions of a serious character at any site, and the dosage required to produce anæsthesia. It is in this respect that ether possesses such great advantages over chloroform, and ethylene over nitrous oxide.

Toxicity of Chloroform

If we turn to chloroform, the first anæsthetic which came into general use, its toxicity expresses itself in several sites. First, we may mention the fall in blood pressure which almost invariably accompanies its exhibition from an early stage in the anæsthesia, and is due to a depression of the vasomotor centre. This is an unavoidable accompaniment of chloroform anæsthesia, and one which must be borne in mind. I think it has been too little regarded in the past, and accounts for deaths which have been ascribed to the operation rather than to the anæsthetic. I have little doubt that it also increases the dangers of post-operative low blood pressure and shock.

Secondly, chloroform depresses the respiratory centre from the first, and this too contributes to its danger in cases which have been suffering prolonged pain, and in whom the respiratory centres have been for some time receiving an undue inflow of nervous impulses travelling over the collateral connections of the pain afferent fibres. Respiratory failure due to overdosage with chloroform is in the lower animals very difficult to combat. I have had cases where the blood pressure was not seriously depressed, and the heart action good, in which chloroform caused a cessation of respiration. Artificial respiration was employed and tended to improve the blood pressure, but the respiratory centre was slow in regaining its activity, and seemed to be aided but little

by such drugs as adrenalin, atropine, strychnine or cardiazol.

The third site of danger during the administration of chloroform is in the heart. I think the best explanation of a great many cases of early deaths under chloroform is that based on Levy's[18] observations on cats, which have been confirmed by other observers in other laboratory animals. Chloroform produces such a change in the cardiac musculature, that the additional action of adrenalin may produce ventricular fibrillation and rapid death. This effect is only produced in light anæsthesia, and when one considers the state of fright or of pain, so commonly present in our patients, both of which may contribute to an abnormal output of adrenalin into the circulation, it is not surprising that an early effect of chloroform may be ventricular fibrillation with sudden death. When we realize that cocaine sensitizes the nerve endings of the sympathetic, rendering them more readily affected by normal amounts of adrenalin, (the pupils for instance dilate when adrenalin is instilled into the conjunctival sac), it is not surprising that after cocaine has been applied locally to a mucous membrane, sudden deaths have occurred during chloroform anæsthesia owing to the absorption of amounts of cocaine not in themselves sufficient to cause symptoms; the chloroform plus the absorbed cocaine has induced a state in which normal amounts of adrenalin caused fibrillation and death. I have known two or three cases where this seemed an adequate explanation.

The fourth site where the action of chloroform is deleterious is the liver. We are all familiar, at least from the literature, with the fatty degeneration which may occur subsequent to a chloroform anæsthesia. This has frequently been ascribed to some of the chloroform undergoing a change in metabolism leading to the intracellular production of such a body as phosgene (carbonyl chloride). It is very difficult to tell whether any such decomposition of the chloroform occurs in the body, on account of the large amounts of chlorides present. It has, however, occurred to us that possibly this difficulty might be surmounted by the employment of bromoform, so similar in chemical composition and reaction to chloroform, and from which under similar conditions carbonyl bromide can be produced. Dr. Lucas and Dr. Brown have, therefore, anæsthetized animals with various doses of bromoform usually given per rectum in oil, and have produced anæsthesia of various depths and duration. In all cases pathological examination has disclosed more or less marked changes in the liver, and the liver damage produced has paralleled as nearly as could be expected the degrees of anæsthesia. In prolonged anæsthesia the changes are often extremely marked, resembling in type those produced by chloroform or carbon tetrachloride. We have also been able to demonstrate an excretion in the urine of an inorganic bromide in considerable amounts up to 2 per cent of the bromine contained in the bromoform given, and also the presence of considerable amounts of inorganic bromides in the tissues. These facts definitely indicate that a portion of the bromoform is broken up in the body and that the cause of this peculiar intoxication may be due to this metabolic decomposition. It is perhaps fair to assume that this type of intoxication occurs also with chloroform and may account for the fatty changes found in the liver and in cardiac muscle. Ethyl bromide undergoes the same type of decomposition as was shown long ago by Dresser,[9] and produces similar liver changes; it is therefore probable that ethyl chloride will have similar effects. The point is being studied in this laboratory, and we have been able, I think, in this experimental work to show the probability of the intoxication produced by this anæsthetic to be dependent in part on its decomposition in the body.

Chloroform then has deleterious effects that are:—

1. Essential in the administration,
   (a) vasomotor centre depression,
   (b) respiratory centre depression,
   (c) intracellular metabolic changes in liver, heart, etc.
2. Due to accessory factors,
   (a) excess adrenalin action with circulation due to pain, or fright, superadded to the normal changes in the cardiac muscle produced by chloroform.
   (b) cocaine sensitizing to adrenalin, added to the chloroform cardiac changes.
3. Due to overdose or lack of care or in debilitated cases, and the narrow margin between necessary and toxic amounts in the blood stream.

Toxicity of Ether

The type of toxic action described for chloroform seems hardly likely to occur with ether, whose chemical decomposition will produce

probably only carbon dioxide and water, and there is little reason to suspect the production of any deleterious chemical substances, though very probably some ether may also be broken up in the body. The toxicity of ether depends on its own inherent chemical or physical properties, or upon changes in metabolism produced by its exhibition, and I hasten to point out that this is probably true of some of the toxic features shown by chloroform. Ether is but slightly toxic to the heart, and depresses the vasomotor centre and the respiration but little, though in overdoses the respiratory centre is affected. The marked depression of the respiratory centre due to ether appears to be more easily overcome than that produced by chloroform. Ether, however, does produce very marked changes in metabolism, as has been shown by Wesley Bourne and Stehle,[4] Leake[14] and other observers. The changes are such as to suggest imperfect oxidations occurring in the body, and it would be interesting to learn whether this was a necessary concomitant of anæsthesia as such. Winterstein and other observers have called attention to the fact that the cells of the central nervous system, (and the same may apply to other tissues), are less able to take up oxygen in the presence of an anæsthetic than they are under normal conditions. The anæsthetic may indeed change both the adsorptive and the chemical characteristics of the cell membrane. This we know occurs under the conditions studied by Warburg on yeast and other cells, and also in the experiments by Lilly on his sensitive iron wires. It is, therefore, readily conceivable that these changes in metabolism are an essential expression of the changes produced in the cells by the presence of the anæsthetic and that the reason why they occur more strikingly with ether is due to the fact that ether is so soluble in water as to permit all the tissue cells in the body to take up relatively large quantities of it.

There is little doubt that one of the disadvantages of ether consists in the pronounced increased secretion of the salivary and bronchial glands. There is no doubt that this is reflexly produced in the main, but this reflex effect seems to be increased by the fact that ether stimulates the central nuclei of bronchial and salivary secretion. The increased secretion occurs not only in the early stages of induction but also during recovery. How far this is the essential factor in the causation of post-operative bronchitis and pneumonia we do not know.

Perhaps the most objectionable feature with ether is the prolonged post-anæsthetic nausea. This is doubtless contributed to by the metabolic upset of which I have spoken, by the fact that such large quantities of ether are absorbed and are given off for a long period of time, and further, by the fact that ether in low concentrations does undoubtedly seem to sensitize medullary centres to an extent that does not occur with chloroform. I may adduce in support the early stimulation of respiration and of blood pressure occurring with ether. Furthermore, I have not been convinced that the methods suggested for combating post-operative nausea with insulin and glucose will achieve the desired results. It is true that this procedure may help to rectify the metabolic upset, but even of this I am not sure, and so far as I know this question has not been studied by the employment of the biochemical methods now available.

To sum up therefore, the deleterious effects of ether are:—

1. Essential,
   (a) increased salivary and bronchial secretions;
   (b) the tendency to upset the metabolism of the body as a whole as expressed by the CO_2 combining power of the blood, and the Ph, and further, by the hyperglycæmia and post-anæsthetic excretion of phosphates.
2. Dangers due to overdosage and a debilitated state of the patient which later may be increased by the metabolic changes.

Nitrous Oxide

As the experiments of Hermann,[11] Kobert,[12] Paul Bert,[2] Bock,[3] show, nitrous oxide has practically no toxic effect on the medullary centres or the heart. It seems to be inherently the least toxic of any of our anæsthetics, but it is undoubtedly the least efficient. Indeed Brown and Lucas[5] have recently published a paper which, taken in conjunction with the work of Greene,[10] I think definitely shows that nitrous oxide as such is an extremely weak anæsthetic and that true surgical anæsthesia cannot be produced by it alone, but only when combined with a certain amount of anoxæmia or combined with other depressants of the central nervous system such as morphine or scopolamine. While it is hard to say that nitrous oxide in itself is at all toxic, yet in looking over

the records of a large general hospital I find that there have been six cases of death from nitrous oxide, and eight deaths from other anæsthetics. Of the deaths under other anæsthetics there seems every reason to believe that in seven instances the anæsthetic was not a large contributing factor, if it contributed at all, the operations in themselves being of an exceedingly dangerous character or the patient in an extremely bad state. Whereas in the deaths occurring during the gas and oxygen anæsthesia, there seems little reason to believe that the state of the patient or the character of the operation was any more responsible for the death than was the anæsthetic. I believe that in most of the cases in which gas-oxygen anæsthesia was given over a prolonged period, death was due to the anoxæmia. As Greene has shown, the hæmoglobin carries only some 80 to 85 per cent of its normal load of oxygen during a nitrous oxide anæsthesia. Leake[14] and his co-workers have pointed out that there is a gradually increasing acidosis. When it is considered that all the cells of the body are probably permeated with nitrous oxide, as it is relatively soluble in water, it will be recognized that the presence of nitrous oxide, may prevent the ready acceptance of oxygen by the tissues, and in consequence of the low saturation of the hæmoglobin produce serious oxygen deficiency in the tissues, and grave disorder in metabolism. I believe the deaths that occur in these cases are due to the heart muscle working for a prolonged period of time in the presence of relatively deficient amounts of oxygen. Nitrous oxide may be said to have in itself no essential deleterious effect, but if not supported by morphine or scopolamine, it always produces an anoxæmia which in many cases is dangerous.

Ethylene

It is interesting to note that with ethylene the metabolic upset is relatively less than with ether, chloroform or nitrous oxide (Leake and Hartmann,[14] Oberhelmann and Dyniewicz[20]), and is probably to be explained in part by the lack of oxygen which occurs in the early stages of deep anæsthesia, and also by a hypothetical inability of the cells to accept oxygen in the presence of an anæsthetic. Though to a somewhat lesser extent than with NO_2, the amount of oxygen required for normal metabolism is decreased, which may be of importance in thyroid cases (Lundy[16]). It will be noted that ethylene does not seem to depress the movements of the intestinal canal to as great an extent as ether or chloroform, and that in consequence one should expect less frequent intestinal paresis after its administration. (Miller[19]). This appears to be confirmed by the data published by Lundy. Post-operative pulmonary complications are less than with ether (Lundy[17]). The dangers of overdosage with ethylene appear to lie firstly in the anoxæmia produced, and secondly in its depressant effect on the respiratory centre and on the vasomotor centre. Respiration ceases before the blood pressure has fallen seriously, at all events in animals, though the blood pressure is less than normal. The heart and its centres seem to be relatively little affected, though sensory reflexes through the cardio-inhibitor centre are definitely reduced. It may, however, be noted that the blood pressure may increase in certain patients and this rise may be harmful (Sise[21]). These experimental observations are confirmed by the work of Lundy,[16] Horsley, Jr.,[12] Meeker,[18] and Allgeyer.[1]

Ethylene appears then to have little essential deleterious effect, and the only secondary ones are due to a relative anoxæmia, and metabolic changes. Danger arising from overdosage are failure of respiration and blood pressure.

Acetylene

As far as may be judged from the literature acetylene can always be given with such ample amounts of oxygen that its exhibition is accompanied by little or no metabolic changes but this point does not seem to have been carefully studied. It does not depress the respiration in anæsthetic doses or even moderate overdoses, but there is a very frequent increase in blood pressure of 20 to 50 mm. (Brandt[5]).

Propylene

Whereas propylene as produced in this laboratory and employed by Brown,[7] Bonham and myself seemed to be the best anæsthetic gas, it was subsequently found, when tanked, to possess undesirable nauseating qualities and to produce indefinite symptoms of intoxication.

This mystery has not been cleared up. Whether there was a difference in the purity of the propyl alcohol used in the two cases, since they were derived from different manufacturers, or whether propylene undergoes an unknown type of polymerization, has not yet been decided. There certainly seems to be in the tanked propylene a relatively less volatile and very odoriferous substance which was not present, at all events in the same quantity, in the propylene we employed. This mystery has still to be elucidated; it is therefore not necessary to say anything further in regard to the toxicity of propylene. It should, however be noted that were this difficulty removed, propylene would possess the advantage, shared also by acetylene, that ample quantities of oxygen be given; indeed, the quantities would be double those ordinarily employed, and in consequence the body would be given every facility for absorbing oxygen, and (Brown and Henderson[7]) the metabolic upset would be found to be smaller in amount.

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A Micro Method for Determination of Nitrous Oxide in Blood[1]

CHARLES KENNEDY.[2] *From the Children's Hospital of Philadelphia and the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*

ABSTRACT

KENNEDY, CHARLES. *A micro method for determination of nitrous oxide in blood.* J. Appl. Physiol. 11(1): 141–142. 1957.—A micro method for the determination of nitrous oxide in blood has been devised which uses the the standard Van Slyke apparatus with a modified extraction chamber. The procedure is essentially the same as that of the original manometric technique except that the volume of the reagents has been reduced, permitting the determinations to be made on 0.2-ml samples of blood. Although slightly more sensitive to changes of room temperature than the macro method, it is comparable in speed and simplicity and is only slightly less precise.

THE QUANTITATIVE DETERMINATION of nitrous oxide in blood has been of importance both in determining the rate of uptake of this gas in the body (1) and in the measurement of the rates of blood flow in various organs of the body (2–4). Inasmuch as serial samples are usually drawn, the volume of blood required for such studies is often considerable, and therefore, to permit investigations in small or anemic individuals a micro procedure has been devised by modifying the generally accepted manometric method (5, 6).

A 10-ml extraction chamber[3] is fitted to the standard Van Slyke manometric apparatus. The constant gas volume, at which all pressures are read, is 0.2 ml, but an additional mark is placed at 0.9 ml. A 2-ml cup at the top is graduated in tenths of a milliliter. To minimize the effect of fluctuations of room temperature, the water jacket is fitted as close to the stopcock as possible. A mercury seal at the junction of the chamber with the 'W' tube was found essential to prevent gas leaks. Kirk ultramicro pipettes, designated 'to contain' 200λ, but recalibrated 'to deliver,' are used to admit the blood sample. The tips of these are fitted with polyethylene tubing to permit tight seating in the cup during admission of the sample. The reagent used is the usual alkaline oxygen absorber which is placed in an oiled, 10-ml syringe.

The procedure for the analysis is as follows: one or two drops of caprylic alcohol are added to the cup and the mercury is lowered half way down the capillary of the chamber stopcock. The excess caprylic alcohol is removed, and the cup is filled with 0.9 ml of distilled water. This is run in, the top stopcock is sealed with mercury and vacuum extraction of the water with the mercury column at the 10-ml mark of the chamber is performed for 3 minutes. During the extraction period the blood sample, with mercury previously added to the syringe, is shaken well, and the pipette is filled to the mark using a rubber adapter. The de-aerated distilled water is allowed to rise to the 0.6-ml mark in the cup, and then is lowered to 0.5 ml in order to draw the mercury from the seal into the chamber. The blood sample is then added, its admission being controlled entirely with the lower stopcock. In order to achieve sufficiently delicate control for this maneuver, the latter must be lubricated with a minimum of grease, all excess having been removed by repeated vigorous flushing of mercury through the narrow slip when the stopcock is barely open. Following admission of the sample, 0.1 ml of the de-aerated water in the cup is let in and the excess in the cup is removed with gentle suction. About 0.5 ml of the oxygen absorber is then added to the cup and a sufficient amount is run in to bring the mercury

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[2] Life Insurance Medical Research Fund Fellow 1951–53.

[3] Made by Mr. J. D. Graham, glass blower, School of Medicine, University of Pennsylvania.

TABLE 1. MANOMETRIC FACTORS FOR CALCULATION OF BLOOD N_2O IN VOLUMES PER CENT USING MICRO PROCEDURE

Temp. analysis ° C.	Factor
20	0.1310
21	0.1305
22	0.1299
23	0.1293
24	0.1287
25	0.1281
26	0.1276
27	0.1270
28	0.1265
29	0.1261
30	0.1257

meniscus to the 0.9-ml mark on the chamber. The top stopcock is sealed with mercury, the mercury column is lowered to the 10-ml mark and the liquid extracted for 3 minutes. Following this, vacuum extraction pressure and temperature are read after gently raising the level of the blood-reagent mixture to the 0.2-ml mark. Subsequent determinations are carried out without washing the chamber, the time required for each analysis being 10 minutes. A blood bank, with the reading corrected for the alteration of water vapor pressure with any temperature change, is subtracted from the reading of the sample, and the difference, multiplied by the appropriate factor, expresses the result in volumes per 100 ml of blood. The factors for denitrogenated blood given in table 1 were calculated using interpolated values for [4] ranging from 0.505 to 0.433 over the temperature range from 20° to 30°C (Kety, unpublished data) and an empirical factor of 1.006.

On comparing values obtained over a range from 0–14 volumes per cent no significant difference was found between the means obtained with the micro method as compared with those of the marco method and the average deviation between the means of triplicate determinations on the same blood samples of both methods was 1.3%. The micro procedure (S.D. = ±0.025) is slightly less precise than the original method (S.D. = ±0.009) since the variance within replicate determinations by the former technique significantly exceeds that of the latter ($P < 0.01$).[5]

The micro procedure was found to be subject to somewhat greater errors resulting from fluctuations of room temperature, probably because the constant gas volume in the micro method is located at the very top of the water jacket. A jacket made to enclose the entire chamber including the stopcock and cup, perhaps, would obviate this difficulty.

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[4] A set of factors, useful in conjunction with the nitrous oxide method for determining cerebral blood flow in which the total inert gas tension is maintained constant, may be obtained by multiplying the factor for any given temperature by 1.04 (Kety, unpublished data, and (6)).

[5] Determined by the Snedecor *F* test.

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Experimental and Epidemiological Investigations on the Effect of Nitrogen Oxides on Lipid Metabolism

Stanisław Kośmider and Adam Misiewicz

Clinic of Internal and Occupational Diseases at the Silesian Medical School, Katowice
(Head: Prof. Dr. W. Zahorski)

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Summary. Experimental and epidemiological investigations were performed with the aim of elucidating whether chronic exposure to nitrogen oxides resulted in impaired lipid metabolism. In experiments on guinea pigs exposed for a period of 6 months to 1 ppm of nitrogen oxides a rise in the level of total lipids and cholesterol accompanied by a fall in the liver cholesterol content was found.

Epidemiological investigations on human subjects chronically exposed to nitrogen oxides revealed a rise in the serum total lipid level accompanied by a rise in the level of β- and γ-lipoproteins and a fall in α-lipoproteins.

The results obtained may indicate that nitrogen oxides exert an arteriosclerotic effect.

Key words: Cholesterol — Total lipids — Nitrogen oxides.

Nitrogen oxides enter the organism through the respiratory tract. The partial penetration into the alveoli causes the damage. After combination with alkalies, nitrates and nitrites arise which may cause general toxic effects when absorbed into the blood stream [12].

The toxicity of nitrogen oxides depends on their concentration and the duration of exposure. A correlation has been shown between the concentration of gaseous atmospheric pollutants and the frequency of circulatory and respiratory diseases [6—8].

It has been demonstrated that nitrogen oxides exert an inhibitory effect on protein biosynthesis and on numerous enzymes in blood and tissues [1, 3, 5, 12].

Impaired lipid metabolism in the lungs of animals exposed to nitrogen oxides is also known [11].

The question has been raised as to whether lipid metabolism is impaired in blood and other tissues in both experimental and human exposure. This problem constitutes the subject of the present paper.

Material and Methods

A. Experimental Investigations

The experiments were performed on 80 male guinea pigs bred in the Central Animal House of the Silesian Medical School. The animals were kept all the time on a standard diet. They were divided into groups.

The control group consisted of 50 guinea pigs weighing 350—530 g (mean 472.0 ± 11.3). The animals were kept in experimental chambers but breathed fresh air.

The intoxicated group consisted of 30 animals weighing 400—590 g (mean 481.0 ± 13.7), exposed for 8 hrs daily for 180 days in chambers of 1 m^3 capacity. Nitrogen oxides in a concentration of 1 ppm were introduced into the chambers. They were generated in a large Kipp apparatus. Constant registration of their concentration was necessary.

Nitrogen oxides were determined colorimetrically with Griss' reagent. The method is based on the absorption of nitrogen oxides in a basic solution giving equal amounts of nitrates and nitrites. Nitrates react with the Griss-Ilosvay reagent to form a pinkish red compound which can be easily determined colorimetrically. The temperature in the chambers was kept between 22 and 23°C, and relative humidity at 65—70%. The amount of air introduced into the chamber was 6000 l per hour, involving 6 changes per 60 min.

In this way it was possible to avoid the accumulation of metabolic waste products from the animals (CO_2, NH_3) [9]. The animals were weighed once 1 week before entering the chamber.

The following parameters were measured: body weight, serum total, free and esterified cholesterol after Chiamori and Hery [2], total lipids and lipidogram after Shwan [10]. After sacrifice, sections of liver were taken, homogenates prepared and total lipids and total cholesterol determined. The results were subjected to statistical analysis.

B. Epidemiological Observations

The investigations were carried out in a group of 70 male workers exposed chronically to nitrogen oxides in a chemical factory. The concentration of the oxides at their places of work ranged between 0.4 and 2.7 ppm. The exposure time was 6 to 8 hrs daily for 4 to 6 years. The age of the exposed workers ranged from 26 to 48 years.

The control group consisted of 80 healthy nonexposed workers in the same age group.

The following parameters were investigated in all the workers: total, free and esterified cholesterol, total lipids, and lipidogram. The methods used were the same as those described above. The results were subjected to statistical analysis with Student's *t*-test.

Results

All the animals exposed to 1 ppm nitrogen oxides survived the 180 days of the experiment. They gained from 11 to 112 g in weight (62.0 ± 23.2 on average). At the same time the weight of the control group increased from 350 to 450 g (395.0 ± 12.4).

Table 1. The level of total, free and esterified cholesterol, total lipids and lipoprotein fractions in serum, and the content of total lipids and total cholesterol in liver homogenates of control and exposed animals

	Control group $\bar{x}_1 \pm S$ (range)	Animals exposed to nitrogen oxides $\bar{x}_2 \pm S$ (range)	$\bar{x}_1 - \bar{x}_2$	Student's t-test
Total cholesterol (mg/100 ml)	45.5 ± 8.72 30.1 — 61.0	53.42 ± 12.2 38.4 — 87.0	−7.9	2.5**
Cholesterol esters (mg/100 ml)	24.2 ± 7.15 12.0 — 39.0	28.0 ± 7.55 16.0 — 48.0	−3.8	1.7*
Free cholesterol (mg/100 ml)	21.5 ± 3.94 16.6 — 29.4	23.3 ± 10.8 8.8 — 49.0	−1.8	0.8
Total lipids (mg/100 ml)	403.0 ± 75.8 240.0 — 570.0	531.2 ± 121.5 275.0 — 730.0	−128	4.3***
α_1- + α_2- lipoproteins (%)	39.6 ± 5.19 30.6 — 50.0	40.0 ± 3.26 35.0 — 46.0	−0.4	0.33
β- + γ- lipoproteins (%)	60.4 ± 5.18 50.0 — 70.0	60.0 ± 3.25 54.0 — 64.0	0.4	0.4
Total lipids in liver homogenates (mg/100 g fresh tissue)	4475.0 ± 1369.4 2500.0 — 7000.0	4608.0 ± 1194.7 3500.0 — 6800.0	−123	0.35
Total cholesterol in liver homogenates (mg/100 g fresh tissue)	270.0 ± 42.4 200.0 — 304.0	238.0 ± 59.9 172.0 — 278.0	−32.0	2.1*

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

The serum of the exposed animals showed a significantly raised total cholesterol level as compared with the controls. Similar results were obtained with cholesterol esters. Free cholesterol tended to be increased but the changes were not significant.

Total lipids were also significantly increased in exposed animals but no changes were seen in the lipidograms.

In liver homogenates prepared from animals chronically exposed to nitrogen oxides the only significant finding was a fall in the total cholesterol level. Total lipids did not differ essentially as compared with controls (Table 1).

Table 2. The level of total, free and esterified cholesterol in serum of the human control group and human subjects chronically exposed to nitrogen oxides, in relation to age

Age (years)	26—36				37—48			
Population	Exposed to nitrogen oxides $\bar{x}_1 \pm S$ (range)	Control group $\bar{x}_2 \pm S$ (range)	$\bar{x}_1 - \bar{x}_2$	t	Exposed to nitrogen oxides $\bar{x}_1 \pm S$ (range)	Control group $\bar{x}_2 \pm S$	$\bar{x}_1 - \bar{x}_2$	t
Number of cases	42	42			28	38		
Total cholesterol (mg/100 ml)	180.0 ± 29.8 143.0 — 230.0	224.0 ± 28.1 160.0 — 280.0	44.0	6.9	214.0 ± 39.9 140.0 — 300.0	265.6 ± 29.6 190.0 — 316.0	51.6	5.9
Statistical significance	$p < 0.001$				$p < 0.001$			
Esterified cholesterol (mg/100 ml)	103.9 ± 24.2 64.0 — 168.0	144.8 ± 19.8 100.0 — 180.0	40.9	8.9	114.0 ± 31.6 62.0 — 197.0	165.6 ± 21.9 105.0 — 215.0	51.6	7.4
Statistical significance	$p < 0.001$				$p < 0.001$			
Free cholesterol (mg/100 ml)	76.0 ± 23.5 32.0 — 132.0	78.7 ± 19.8 35.0 — 120.0	2.7	0.6	99.9 ± 30.9 38.0 — 173.0	103.0 ± 24.8 48.0 — 195.0	3.1	0.6
Statistical significance	not significant				not significant			

Table 3. Total lipids and lipoprotein fraction in serum of the human control group and human subjects chronically exposed to nitrogen oxides in relation to age

Age (years)	26—36				37—48			
Population	Exposed to nitrogen oxides $\bar{x}_1 \pm S$ (range)	Control group $\bar{x}_2 \pm S$ (range)	$\bar{x}_1 - \bar{x}_2$	t	Exposed to nitrogen oxides $\bar{x}_1 \pm S$ (range)	Control group $\bar{x}_2 \pm S$ (range)	$\bar{x}_1 - \bar{x}_2$	t
Number of cases	42	42			28	38		
Total lipids (mg/100 ml)	718.8 ± 24.5 500.0 — 1000.0	582.7 ± 80.3 495.0 — 750.0	136.1	3.2	754.7 ± 89.2 500.0 — 900.0	598.9 ± 73.2 460.0 — 780.0	155.8	7.6
Statistical significance	$p < 0.01$				$p < 0.001$			
Lipidogram (%)								
$\alpha_1 + \alpha_2$	19.0 ± 8.0 6.0 — 39.0	31.1 ± 4.5 24.0 — 40.0	12.1	8.9	21.2 ± 7.0 13.0 — 40.0	30.9 ± 4.8 19.0 — 40.0	9.7	6.5
Statistical significance	$p < 0.001$				$p < 0.001$			
$\beta + \gamma$	80.9 ± 8.0 61.0 — 94.0	68.6 ± 4.5 60.0 — 74.0	12.3	7.1	78.8 ± 7.2 72.0 — 87.0	69.0 ± 4.8 60.0 — 81.0	9.8	6.5
Statistical significance	$p < 0.001$				$p < 0.001$			

In the serum of human subjects chronically exposed to nitrogen oxides a fall of all the cholesterol fractions was observed. The most distinct changes affected total and esterified cholesterol. In the control group an increase of total and esterified cholesterol with age was seen. The same applied to the exposed subjects. The differences between the exposed and unexposed subjects were significant in the different age groups (Table 2).

The concentration of total lipids increased significantly as compared with that in the control group. Within the lipidogram the following statistically significant changes were observed in subjects exposed to nitrogen oxides: decreased $\alpha_1 + \alpha_2$ fraction and increased $\beta + \gamma$ fraction (Table 3).

As the level of lipids also increases with advancing age, an analysis was made to determine whether the results obtained were due to the effect of nitrogen oxides or to lipid metabolism alterations connected with age.

It was demonstrated that the level of total lipids also increased with age in exposed subjects. Nevertheless, the increase was significantly greater than in the control group.

Discussion

The experiments performed demonstrated that chronic exposure to nitrogen oxides caused disturbances in lipid metabolism in animals. A rise was seen in the levels of total lipids and of all cholesterol fractions in serum. At the same time a fall in the cholesterol level took place in the liver, the site of cholesterol synthesis. It may be assumed that hypercholesterolemia brought about results by way of a diminished cholesterol degradation or increased production in the liver accompanied by rapid liberation into the blood stream. The esterification process remained normal. On the other hand the level of total lipids increased in both serum and liver.

The epidemiological experiments performed in men chronically exposed to nitrogen oxides also showed impaired lipid metabolism. It was even more pronounced than in animals although differing slightly in the biochemical picture.

In men, changes in lipoproteins were observed in addition to an increased total lipid concentration in serum. A decrease of the α-fraction with a corresponding increase of the β- and γ-fractions was found.

Statistical analysis showed that the increase in the level of serum lipids in men depended not only on age but also on the influence of nitrogen oxides.

It is interesting to note that inversely to animals, exposure to nitrogen oxides in men caused a drop in the level of cholesterol. This may be caused by differences in lipid metabolism between men and guinea pigs. In addition, the duration exposure was several years in men whereas in animals it was only several months.

The effect of impaired lipid metabolism connected with exposure to nitrogen oxides on blood vessels and the possible effect on the intensity of arteriosclerosis needs further investigation.

Conclusions

1. Chronic exposure to nitrogen oxides causes impaired lipid metabolism both in animals and humans.
2. Exposure to nitrogen oxides results in raised cholesterol and total lipids levels in serum of animals.
3. Chronic exposure to nitrogen oxides in humans results in increased levels of serum total lipids, β- and γ-lipoproteins as well as in a fall of α-lipoproteins.
4. The problem of a possible effect of nitrogen oxides on arteriosclerotic processes needs further investigation.

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Prof. Dr. med. Stanislaw Kośmider
Klinika Chorób Wewnętrznych
i Zawodowych
Sl. Ak. Med.
ul. 3 Maja 15
Zabrze, Poland

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Non-medical use of nitrous oxide:

A preliminary report

By Edward J. Lynn, MD
Mary James
Robert Dendy, MA
Lance A. Harris, MA
Richard G. Walter, PhD
All of East Lansing

Introduction

Ever since Sir Humphry Davy's treatise on the use of nitrous oxide[1] suggesting that the gas was not only an anesthetic/analgesic agent but that it provided pleasurable effects as well, its underground use has allegedly increased. Stimulated by an increasing number of anecdotal reports on the non-medical use of nitrous oxide (N_2O) a research team composed of members of the departments of Psychiatry, Psychology, the Counseling Center and the Drug Education Project of Michigan State University, the Drug Education Center of East Lansing and the Governor's Office of Drug Abuse was organized to examine several aspects of the N_2O scene.

We learned by surveying the literature that there were few publications that addressed themselves to the non-medical use of N_2O.[2-6] The few reports concerning the effects of N_2O on perception and cognitive functioning[7-12] were not carried out in a fashion approximating the usual street use of the drug. We set about to:

1. Survey Mid-Michigan to assess the prevalence of use of N_2O.
2. Establish a safe method of using the agent that approximates the usual use of N_2O.
3. Assess cognitive functioning during various time periods after volunteers became "high" on N_2O.

1. Survey

A. *Method*

Members of the Drug Education Center as well as drug users in the community were asked to informally survey the "drug scene" to determine if and how N_2O was being used, and if its use was increasing.

B. *Preliminary Findings*

N_2O is being used in mid-Michigan in various ways.

Several individuals reported having been to parties where a professional (doctor, nurse, dentist, inhalation therapist, researcher) provided N_2O for the participants to "turn on." There were many who noted using the N_2O contained in aerosol cans (either whipped cream or motor starter) and others who tapped the gas from large tanks in restaurants used for preparing whipped cream. At the recent Goose Lake Rock Festival the gas was widely sold for 25 cents a balloon. We also made contact with a mystical-religious group that used the gas to accelerate arriving at a transcendental-meditative state.

Few used elaborate equipment (i.e., N_2O-O_2 mixes with sophisticated valves and masks). Most used balloons or plastic bags and either held a deep breath of pure N_2O or rebreathed it. No adverse effects were reported by any of the more than one hundred individuals surveyed. There was a general impression that the use of the gas was on the increase.

2. Pilot Study

A. *Method*

Ten volunteers—seven men and three women (ages 21-42) were used. Five had no previous drug experience other than alcohol or prescription drugs ("naive") and five had previous drug experiences including marijuana, LSD, mescaline, heroin and others ("sophisticated"). All subjects were given complete physical exams and hematocrit determinations were made. The subjects were asked to take a deep breath of pure N_2O from a balloon or rebreathe it. Onset of peak effects was timed and subjective reports were gathered.

B. *Results*

All of the subjects reported an exhilarating "high" within 15-30 seconds after inhalation. The initial peak effects lasted from 2-3 minutes and were described as pleasurable by all. All described a "head rush"—tingling or warmth around the

Doctor Lynn is assistant professor of psychiatry in the College of Human Medicine; Miss James is with the Drug Education Project; Mr. Dendy is with the Counseling Center; Mr. Harris is with the Department of Psychology and Doctor Walter is an assistant professor of business finance in the College of Business. All are with Michigan State University.

face and head and auditory illusory or hallucinatory phenomena. Rebreathing the gas prolonged the peak.

All of the subjects described a state of well being that lasted beyond the peak from minutes to hours (somewhat longer for the drug-sophisticated group than the drug-naive group). A "reverse tolerance" was described by most – more intense or prolonged experiences with subsequent trials of the same dosage. Several volunteered that headaches, shoulder pain and mild depression was ameliorated during the experience and did not return.

3. Cognitive Functioning

A. *Method*

Three tests were utilized to assess cognitive functioning before, during and at five and 10 minute periods after the onset of the N_2O effects. These included the digit symbol test (DS) from the Wechsler Adult Intelligence Scale[13] with parallel forms, the symbol analogies test (SA)[14] with parallel forms, and an addition test (AD) with parallel forms. The subjects were 12 men and 12 women (ages 19-30), half of whom were drug naive and half sophisticated. Four periods of test administration were scheduled at 24 hour intervals. The temporal order of administration, (during a "high," post-five minutes, post-10 minutes) were counterbalanced so that all subjects received all three tests in all three temporal orders. The data were then subjected to analysis of variance.

B. *Results*

The entire group (n=24) demonstrated significant cognitive defects ($\alpha < .01$) during the "high" for the DS and AD but not the SA. Significantly lower performances were observed for the number correct, number tried and percent correct. There were no significant differences between pre-tests and tests during the five and 10 minute periods. The naive group showed no significant performance differences from the sophisticated group save for one area – the drug users did significantly better on SA testing at pre- and post-five minutes ($\alpha = .04$).

Subjective responses were similar for this group of 24 as the pilot group of 10. More of the naive subjects tended to report somatic sensations (warmth, tingling) and the sophisticated subjects tended to report "psychedelic" experiences – comparing the high with that achieved by other drugs. Adverse experiences were minimal (seven naive, one sophisticated) and included transient nausea, bloating, pressure in the head, dry throat and mild suspiciousness.

Discussion

The non-medical use of N_2O, although not rampant, seems to be on the increase in mid-Michigan. The usual use of the gas (from a balloon) appears to be a safe procedure but one needs to assess the decrease in cognitive functioning, as measured by DS and AD testing, which occurs during a high. These defects, however, are transient and functioning returns to normal within five minutes. Users should be alerted to the possibilities of impaired performance during the peak effects of the drug. There seems to be little difference in the effects on cognitive functioning when drug-naive subjects are compared with drug-sophisticates. Subjective responses also seem to be similar with greater somatic sensations among the naive subjects. The drug users tend to have longer after-effects which may have more to do with set and setting than with the physiological effects of N_2O.

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CHRONIC EXPOSURE TO ANAESTHETIC GASES IN THE OPERATING THEATRE AND RECOVERY ROOM

P. NIKKI, P. PFÄFFLI, K. AHLMAN and R. RALLI

FROM THE DEPARTMENT OF OPHTHALMOLOGY, UNIVERSITY CENTRAL HOSPITAL AND THE INSTITUTE OF OCCUPATIONAL HEALTH, HELSINKI, FINLAND

ABSTRACT

The ambient concentrations of halothane measured in 6 operating theatres and 3 recovery rooms averaged 13.9 ppm and 2.8 ppm, respectively. The corresponding values for nitrous oxide were 929 ppm and 146 ppm. A scavenging system reduced the halothane present in the atmosphere of the operating theatres to 0.85 ppm and that of nitrous oxide to 135 ppm, the reduction of the former being more than 90 % and that of the latter about 85 %. The total ventilation measured in all operating theatres and recovery rooms varied from 0—2.8 times/volume/hr. The need for effective ventilation in both recovery rooms and operating theatres in addition to a scavenging system in the operating theatres is emphasized.

KEY WORDS: NITROUS OXIDE; POLLUTION; OPERATING ROOM; RECOVERY ROOM

INTRODUCTION

Several recent studies have shown small concentrations of halothane and nitrous oxide in the air of operating theatres (9, 10, 11, 19), and the potential hazards of anaesthetic gases to personnel chronically exposed have been discussed.

Different devices for eliminating the overflow of gases from the operating theatres have been described (3, 6, 12, 18, 19, 22). Recently, Whitcher et al. (21) made a comprehensive survey of halothane concentrations in the operating theatres and proved the efficiency of their gas scavenging system.

Other opportunities for personnel to be exposed to anaesthetic gases still exist. Except for effective ventilation there is no practical method for eliminating anaesthetic gases exhaled by the patients from the recovery rooms. We therefore decided to study the ambient concentrations of halothane and nitrous oxide in the recovery rooms and, in addition, to measure them in operating theatres with and without the scavenging system.

METHODS

Measurements of the efficiency of the ventilation and the ambient concentrations of halothane and nitrous oxide were made in 6 operating theatres and 3 recovery rooms of 3 hospitals in Helsinki. Two of the recovery rooms were located away from their operating theatres and one was close to them.

Measurement of ventilation

The ventilation in operating theatres and recovery rooms was studied by measuring either the intake or the blow-off of the air. The devices used in the measurements of the ventilation were an Alnor velometer (Illinois Testing Laboratories, Inc., Chigaco, USA) and a thermoanemometer (Wallac, Turku, Finland). Both devices are based on measuring the velocity of air flow.

Measurement of ambient concentrations of halothane and nitrous oxide

Air samples were collected during consecutive 30 minute periods in plastic laminated bags

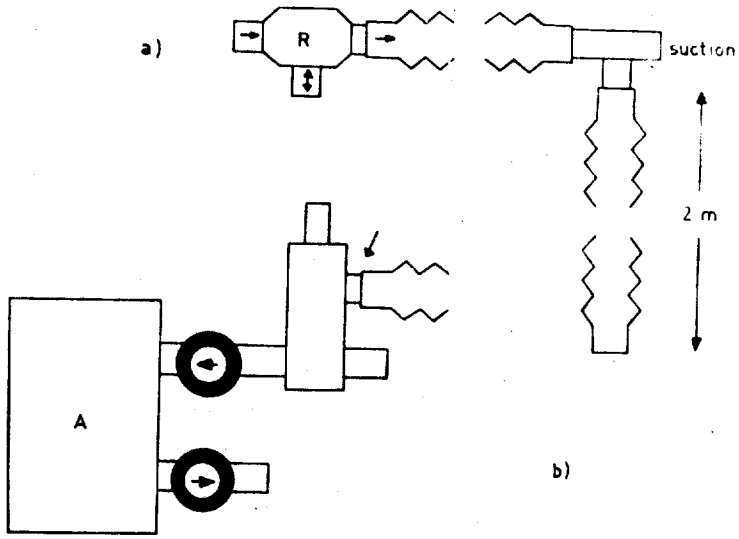

Fig. 1 a. A non-rebreathing system. The outlet part of the Ruben valve (R) is connected through a corrugated tube with a wall suction (see text).
Fig. 1 b. A partial rebreathing system with an absorber (A). The overflow of gases is conducted into the wall suction (as described in Fig. 1 a) through the exhaust pipe (arrow) of the expiratory valve (details in Fig. 2).

(15). The air was blown into the bag with a battery driven pump (Permissible Air Sampling Pump, Approval No. 2F-2004, Mines Safety Appliances Co. Pittsburgh, USA). Each sample represented a mean concentration of halothane and nitrous oxide during each period of sampling. The samples, both in operating theatres and in recovery rooms, were either taken as samples of »general» air, or as »individual» samples. The former were collected in the operating theatres at a height of 150 cm and at a distance of 150 cm from the expiratory part of the anaesthetic circuit, and the latter from the breathing zone of the anaesthetist or anaesthesia nurse to be studied. In recovery rooms the arrangement was similar; samples of general air were taken about 150 cm from the mouth of a patient and at a height of 150 cm, and individual samples as described earlier. The collecting of samples continued through the working day both in operating theatres and recovery rooms from 8.30 a.m.—3 p.m. The operations were routine daily work for an ophthalmic, an orthopaedic, and a general surgical hospital. The anaesthetic systems used were either semi-open or semi-closed systems with gas flows varying from 4—10 l/min. Nitrous oxide concentration was usually 70 % and that of halothane from 0.5 to 1.5 %. Both controlled and spontaneous ventilation was used.

Elimination of overflow of anaesthetic gases

In scavenging the overflow of anaesthetic gases we have used a method previously described by Nikki and Ralli (13). In a nonrebreathing system with a flow of 5—8 l/min, either by using a Ruben-valve or the Jackson-Rees' modification of Ayres T-piece for small children, the outflow of gases was conducted into a wall suction of approximately 10 l/min

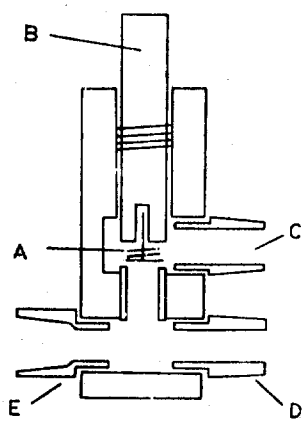

Fig. 2. A valve, B adjusting, C exhaust pipe, D and E inlet and outlet pipes.

capacity. A side branch (a corrugated tubing with a length of about 200 cm) was necessary to avoid subatmospheric pressure in the anaesthetic system (Fig. 1a). In a partial rebreathing system the overflow of gases was conducted into the suction through an expiratory valve (Fig. 1b) with a cross section as described in Fig. 2. (The valve is manufactured commercially by Chr. Nissen Oy, Helsinki, according to the authors' drawings.)

Analysis of the air samples

The samples were analysed using a gas chromatograph: reference gases were halothane and nitrous oxide diluted with air in laminated bags. The details of the gas chromatographic set-up are given below:

TABLE 1

Concentrations of halothane and nitrous oxide in operating theatres.

Sample	Halothane (parts per million, v/v)				Nitrous oxide (parts per million, v/v)			
	Number	Mean	SD	Range	Number	Mean	SD	Range
General no scavenging	34	13.05	7.62	2.3 —30.7	28	880	664	90 – 2750
Individual no scavenging	44	14.09	12.48	2.4 —57.0	34	971	662	60 – 2600
All samples no scavenging	78	13.85	10.43	2.3 —57.0	62	929	659	60 – 2750
All samples with scavenging	27	0.85	0.95	< 0.01— 1.9	28	135	191	25 – 380

TABLE 2

Concentrations of halothane and nitrous oxide in recovery rooms.

Sample	Halothane (parts per million, v/v)				Nitrous oxide (parts per million, v/v)			
	Number	Mean	SD	Range	Number	Mean	SD	Range
Recovery rooms (2) located apart from operating theatres	18	2.31	0.85	< 0.01—3.6	18	120	99	< 5—430
Recovery room (1) close to operating theatres, (when no scavenging in op.th.)	14	3.38	1.78	0.8 —8.2	10	192	136	37—500

For halothane

Gas chromatograph:	Perkin Elmer F6 and F11
Column:	10 % XF 1150 on Chromosorb P, 60/80 mesh. Dimensions 2 m × 4 mm I.D.
Column temperature:	60 C
Carrier gas:	Nitrogen, flow 30 ml/min
Detector:	Flame ionization or electron capture
Sample:	Manual injection of 1 ml of gas sample through gas sampling valve.

For nitrous oxide

Gas chromatograph:	Perkin Elmer F11
Column:	Molecular sieve BX, 30 60 mesh. Dimensions 2 m 1 mm I.D.
Column temperature:	110 C
Carrier gas:	Nitrogen, flow 30 ml min
Detector:	Electron capture
Sample:	Manual injection of 0.1 ml or 5 ml of gas sample through gas sampling valves.

RESULTS

Operating theatres

The mean concentration of halothane in the operating theatres tested was 13.85 ± 10.43 ppm (parts per million) (X ± SD) and that of nitrous oxide 929 ± 659 ppm (Table 1). No difference was found when the samples were either collected at a height of 150 cm and at a distance of 150 cm from the expiratory part of the anaesthetic circuit (samples of general air), or from the breathing zone of the anaesthetist or anaesthesia nurse (individual samples). The scavenging system reduced the mean ambient concentration of halothane to 0.85 ± 0.95 ppm and that of nitrous oxide to 135 ± 191 ppm, the reduction being 93.9 % and 85.4 %, respectively.

Recovery rooms

The ambient concentrations of halothane in 3 recovery rooms averaged 2.77 ± 1.42

TABLE 3

Ventilation in the operating theatres and recovery rooms studied (See Table 1).

Spaces where measurements were made	Number of operations during which the air concentrations were measured	Volume of operating theatres m^3	Floor area of operating theatres m^2	Ventilation m^3/hr	Ventilation × room vol/hr	System of ventilation
A 1 *	7	96	30	30	0.3	Three disk valves, natural ventilation
A 2	4	96	30	40	0.5	Three disk valves, natural ventilation
A 3	9	57.5	18	160	2.8	A large disk valve, natural ventilation
B 1	2	90	35	90	1.0	Mechanical fresh air intake
B 4	2	125	45	75	0.6	A disk valve, natural ventilation
C 1	2	57	18	10	0.2	A disk valve, natural ventilation
A r		108	36	125	1.2	Two disk valves, natural ventilation
B r		108	40			No ventilation system
C r		40	13	10	0.3	A disk valve, natural ventilation

* Hospital: A, B and C; operating theatres: 1, 2, 3 and 4; recovery rooms: r

TABLE 4

Effect of ventilation on ambient concentrations of anaesthetics.

	Halothane (parts per million, v/v)					Nitrous oxide (parts per million, v/v)				
	Number	Mean	SD	Median	Range	Number	Mean	SD	Median	Range
Theatre (B 1) with mechanical ventilation (ventilation amounts as in Table 3)	17	4.08	2.52	3.6	2.4—13.4	17	498	385	490	60—1690
Theatre (A 1) with natural ventilation (ventilation amounts as in Table 3)	9	11.80	4.75	126	4.7—18.5	9	821	325	870	360—1400
	$p < 0.001$					$p < 0.01$				

ppm and that of nitrous oxide 146 ± 115 ppm (Table 2). The recovery room close to the operating theatres indicated a slightly higher concentration of both halothane and nitrous oxide than the two recovery rooms which were apart from the operating theatres. Ambient concentrations of anaesthetic gases in recovery rooms during the measuring days did not seem to increase. (Preliminary results have been published in Br J Anaesth (16).)

Ventilation in operating theatres and recovery rooms

The volume of the operating theatres varied from 57 to 126 m^3 and ventilation volume/hr from 0.2 to 2.8. In one of the recovery rooms there was no ventilation system at all, and in the two others ventilation values varied from 0.3 to 1.2 (Table 3).

Gas concentrations in operating theatres in regard to their volume and ventilation

In an operating theatre with a mechanical air intake system (1.0 × room vol/hr) the ambient concentration of halothane averaged 4.08 ± 2.52 ppm, whereas in an operating theatre with less effective ventilation (0.3), the corresponding concentration of halothane was 11.8 ± 4.75 ppm; the latter thus was significantly higher. The same difference was observable in regard to nitrous oxide values (Table 4). The volume of the operating theatre seems to be important, too. A theatre with a small volume (57 m^3) contained a significantly higher concentration of halothane, 16.00 ± 6.65 ppm, although the ventilation in the latter was less effective (0.3 versus 2.8).

DISCUSSION

The average concentration of ambient halothane in the present study was about twice, and the concentration of nitrous oxide about 6 times as high as those reported before (11, 21). This difference is presumably caused by the ineffective ventilation in our operating theatres. Ineffective ventilation, unfortunately, seems

TABLE 5

Effect of volume of the operating theatre on ambient concentration of anaesthetics.

	Halothane (parts per million, v/v)					Nitrous oxide (parts per million, v/v)				
	Number	Mean	SD	Median	Range	Number	Mean	SD	Median	Range
A large theatre (B 4, 125 m^3)	15	10.74	6.36	10.2	2.6—24.0	15	1241	529	1240	90—2000
A small theatre (A 1, 57 m^3)	19	16.00	6.65	15.8	8.8—27.2	13	1426	893	800	595—2750
	$p < 0.05$					N.S.				

to be common in many operating theatres. As Whitcher et al. stated in their comprehensive article (21), the atmospheric pollution of operating theatres with anaesthetic gases is present and causes a serious problem. The possible hazard of halothane to the personnel has been widely discussed by them (21). Especially the high abortion rates among anaesthetic personnel reported by Askrog and Harvald (1) and Cohen et al. (4) have to be taken seriously. Since almost all inhalational anaesthetics, e.g., nitrous oxide (20, 8), are known to cause teratogenic effects in experimental animals, it is necessary to eliminate all volatile anaesthetic agents from the operating theatres (4). Besides teratogenic activity, high concentrations of nitrous oxide are known to be haematotoxic for experimental animals (7). This effect may be accentuated by leucocytosis (14). No evidence of the possible haematotoxicity of small concentrations of nitrous oxide exists.

In the operating theatres to be studied, a temporary scavenging system was used (13). It reduced the ambient halothane concentration by more than 90 % and that of nitrous oxide by about 85 %. The reduction of halothane was comparable to that of Whitcher et al. (21). Furthermore, we found a decrease of halothane in the endtidal samples from anaesthetists and anaesthesia nurses when the scavenging system was used (17).

The average concentration of halothane in the 3 recovery rooms was about three times as high as that in operating theatres with scavenging, whereas the average concentration of nitrous oxide was about the same in recovery rooms and in operating theatres with scavenging). This finding is probably caused by the slower elimination of halothane than nitrous oxide from the patients. Further, recovery rooms indicated even concentrations of anaesthetic gases during all hours (9—15). The recovery room located close to the operating theatres showed, however, a slightly higher concentration of both halothane and nitrous oxide than those located separately. Some traces of anaesthetic gases may thus move from operating theatres to the recovery room. It would therefore be wise to locate the recovery room apart from operating theatres, when new operating theatres are planned. An effective ventilation, especially in recovery rooms, is necessary, since the scavenging system in recovery rooms seems rather difficult to arrange.

All the pollution problems are not solved with these procedures. There is no simple outlet in each ventilator, for the scavenging system. When anaesthesia with a mask is used, high concentrations of anaesthetic gases may, at least during induction, escape into the atmosphere. The scavenging system described above is a temporary solution, since wall suction is needed for other purposes. We are therefore preparing a new one with an explosion-proof motor to create the suction effect.

In conclusion we recommend:

1. Effective ventilation, at least 20—25 times/volume hr.
2. A practical and explosion-safe scavenging system, usable in all operating theatres.
3. The use of small gas flows in the anaesthetic circuit.
4. Installation of gas outlets for scavenging in all ventilators.

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Address: P. Nikki, M. D.
Department of Ophthalmology
University Central Hospital
Haartmaninkatu 4
00290 Helsinki 29
Finland

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LEUCOPENIC EFFECTS OF PROLONGED NITROUS OXIDE TREATMENT

BY

GEOFFREY D. PARBROOK

Department of Anaesthetics, Royal Infirmary, Aberdeen, Scotland

SUMMARY

Studies of the leucopenic effects of nitrous oxide were made in rodents, with and without surgical wounds. The animals were exposed to one of the following atmospheres: (1) air; (2) 25 per cent nitrous oxide; (3) 60 per cent (rats) or 40 per cent (mice) nitrous oxide; the oxygen percentage was maintained at 21 per cent in each case. A leucopenic effect and suppression of the normal leucocytosis to trauma was seen with all concentrations of nitrous oxide after 2 days' treatment. The effect of 60 per cent nitrous oxide was greater than 25 per cent nitrous oxide after 7 days but the difference was less pronounced in the first 4 days. The leucopenic effect was greater in animals with surgical wounds. Granulopenia was present after 3 days, but not after 1 day of nitrous oxide, and persisted for at least 3 days after completion of treatment. The safety of nitrous oxide therapy and its possible mechanisms of toxicity are discussed.

Long-term nitrous oxide therapy has been recommended for postoperative sedation and pain relief (Petrovsky and Yefuni, 1965; Pugachev and Kozhin, 1965), and also as an aid in prolonged hypothermia (Roth, 1963). In these reports the risks of leucopenia were minimized by frequent blood counts and were weighed against the excellent analgesia and sedation from nitrous oxide. The experimental studies to be described were made to investigate the leucopenic effect of the gas with a view to establishing the relative safety of continuous nitrous oxide therapy.

TECHNIQUE

Norwegian hooded rats were used as the experimental animal for these studies apart from one study in mice. The ventilation units described in the previous paper (Parbrook, 1967) were adjusted in each trial to provide the following atmospheres:

(1) air (controls);
(2) 25 per cent nitrous oxide, 21 per cent oxygen and 54 per cent nitrogen;
(3) 60 per cent nitrous oxide, 21 per cent oxygen and 19 per cent nitrogen.

In each trial, the animals were divided into three groups and each group was exposed to one of the above mixtures. In the case of mice, 40 per cent nitrous oxide was used in place of 60 per cent in group 3.

Administration of a general anaesthetic can affect the white cell count and was not found to be necessary for the taking of blood samples; these were obtained from the tail tip in rats and by retro-orbital puncture (Pettit, 1913; Riley, 1960) in mice. Total white blood cell counts were performed, and blood films were stained with Leishman's stain, according to the techniques described by Dacie and Lewis (1963). The rats were identified by means of an ear punch code, and mice by means of a code with coloured dyes.

RESULTS

Fourteen days treatment with nitrous oxide.

In this trial 36 rats were divided into three groups of 12 and each group was exposed to one of the following mixtures: (1) air; (2) 25 per cent nitrous oxide; (3) 60 per cent nitrous oxide.

In 2 rats in each group a mild inflammatory reaction developed in the tail and prevented completion of serial readings. As the initial readings in these rats agreed closely with the mean for the group concerned it was considered justified to omit these incomplete results from the statistical analysis. The results in the three groups of 10 rats are given in the graph with 95 per cent confidence limits (fig. 1).

A leucocytosis was revealed in the control group and was attributed to inflammatory and stress

response to the tail trauma. Both in the control group and in the 25 per cent nitrous oxide group, treatment was continued for 14 days; the results showed a significantly lower white cell count in the nitrous oxide group than in the controls in all tests, the earliest counts in this trial being made after 4 days of treatment.

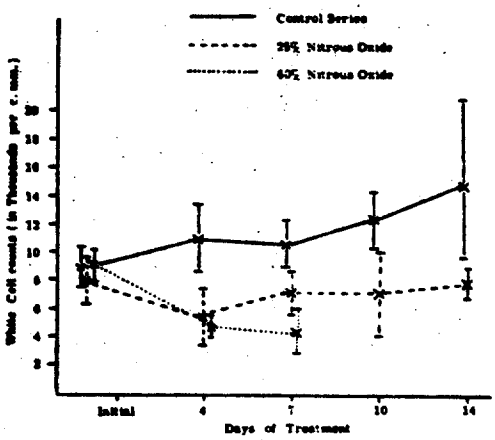

FIG. 1

The graph indicates the mean white cell counts and 95 per cent confidence limits for three comparable groups, each of 10 rats, exposed to air or to one of two concentrations of nitrous oxide. The leucocytosis in the control groups was attributed to tail trauma from repeated taking of blood samples.

The rats treated with 60 per cent nitrous oxide also had lower white cell counts than the controls on the 4th day, and on the 7th day the counts were significantly lower in the 60 per cent group than those in the rats treated with 25 per cent nitrous oxide ($P<0.05$). During treatment with 60 per cent nitrous oxide the animals remained in satisfactory health until the 7th day when 2 animals which had leucopenia appeared ill and a third died showing marked leucopenia (1,250 cells/cu.mm). It was not considered justified to prolong treatment in this group and the animals were destroyed.

White cell counts in the presence of a surgical wound.

In considering the use of nitrous oxide for postoperative pain relief it seemed wise to repeat these results in a comparable series of animals with incised surgical wounds. It appeared possible that in the presence of a wound the gas might prove more toxic to the bone marrow, since healing is associated with a leucocytosis. In view of the serious toxicity of nitrous oxide after 7 days' exposure as shown in the previous trial, the duration of treatment was restricted to 4 days using 24 rats in three groups of 8. Preliminary white cell counts were taken and a 4-cm-long incised wound was made during halothane and oxygen anaesthesia using anaesthetic apparatus described elsewhere (Parbrook, 1966); Michel clips were used to close the wound.

Because of variation in the total white cell counts between different rats, minor changes in counts can be assessed more satisfactorily using the changes in counts in the individual rats. The means of the changes in the white cell counts are given in table I, together with the comparable readings in the rats of the previous trial at 4 days. It is seen that the white cell counts have increased significantly in both the "wounded" and "non-wounded" controls but there is a significant lowering of the counts in all groups of treated rats. While there appeared to be a greater depression of the total white cell count with 60 per cent nitrous oxide than with 25 per cent, these differences were not significant ($P>0.2$ both in rats with and without wounds).

If all the rats treated with nitrous oxide were compared according to whether or not wounded, those with wounds showed significantly greater depression of the white cell counts. Further evidence of increased toxicity of nitrous oxide in the animals with wounds was indicated by the subsequent progress of these rats. In the case of rats with wounds there appeared to be a higher morbidity as 4 rats died or appeared ill and had to be destroyed within 2 days of completion of treatment. It was noted that all these animals had counts below 2,500/cu.mm whereas animals with counts above 2,500/cu.mm remained in satisfactory health.

Effects of nitrous oxide therapy of under 4 days duration.

Two further trials were conducted with rats with wounds to indicate the effects of nitrous oxide treatment over the first 3 days. In the first trial white cell counts were taken initially and after

TABLE I

Mean changes in white cell counts (per cu.mm) after 4 days of inhaling air or nitrous oxide in 25 or 60 per cent concentration.

	Controls (air)			25 per cent nitrous oxide			60 per cent nitrous oxide			Analysis of variance		
	N	$\bar{x}$	SE	N	$\bar{x}$	SE	N	$\bar{x}$	SE	F	d.f.	P
Rats without wounds	10	+2,200†	969	10	−2,570*	984	10	−4,125†	672	13.8	2:27	<0.0005
Rats with wounds	7	+5,529*	1,869	8	−5,494†	882	7	−6,579†	638	29.3	2:19	<0.0005

$\bar{x}$=mean of changes in white cell counts. N=number of animals in each group.
SE=standard error. * Probability, P<0.05. † P<0.01.

Comparing all treated non-wounded with treated wounded animals, the increased depression of the count in the latter is significant (t_{22}=3.26; P<0.01).

TABLE II

Mean changes in white cell counts (per cu.mm) after 1, 2, 3 and 4 days in rats with wounds.

Duration of treatment (days)	Controls (air)			25 per cent nitrous oxide			60 per cent nitrous oxide			Analysis of variance		
	N	$\bar{x}$	SE	N	$\bar{x}$	SE	N	$\bar{x}$	SE	F	d.f.	P
1	6	+1,342	1,667	6	−2,675*	922	6	−2,783	1,334	3.1	2:15	<0.1
2	14	+ 929	719	14	−2,004†	569	13	−3,369†	867	9.2	2:38	<0.001
3	6	+2,325	1,502	6	−2,933*	1,047	6	−4,092*	1,569	7.8	2:15	<0.01
4	7	+5,529*	1,869	8	−5,494†	882	7	−6,579†	638	29.3	2:19	<0.0005

$\bar{x}$=mean of changes in white cell counts. N=number of animals in each group.
SE=standard error. * Probability, P<0.05. † P<0.01.

The rats at each day are different sets except for those which were assessed at 1 and 3 days after the start of the experiment. The counts were made 2 hours after treatment in the "1 day" results and 18 to 20 hours after removal from the atmospheres in the results of the 2, 3 and 4 days.

Duration of treatment. Analysis of trend, in the 2-, 3- and 4-day results:
Controls: the increasing leucocytosis is significant; t_{21}=2.77; P<0.02.
25 per cent nitrous oxide: the increasing leucopenia is significant; t_{22}=3.38; P<0.01.
60 per cent nitrous oxide: the increasing leucopenia is significant; t_{22}=3.53; P<0.01.

1 and 3 days treatment; in the second trial the white cell counts were measured initially and after 2 days of nitrous oxide treatment. The composite results are shown, with those of the previous trial, in the histogram (fig. 2) and in table II.

The control rats showed increases of white cell counts which reached significance after 4 days. A significant depression of the counts was recorded after 2, 3, and 4 days with both 25 and 60 per cent nitrous oxide, and after 1 day in 25 per cent nitrous oxide treated rats.

The difference between rats treated with 25 and 60 per cent nitrous oxide is small compared to the marked differences in the response of the controls and treated animals. The histogram is suggestive of an overall tendency for 60 per cent nitrous oxide to be more toxic than the 25 per cent concentration between the 2nd and 4th days of treatment, but the difference between the two treatments is not significant at any individual day.

Comparison of the results of all four durations of treatment was not possible as the white cell

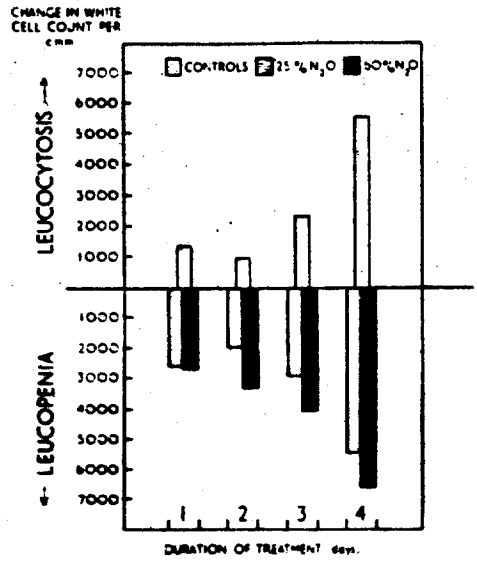

FIG. 2

The histogram indicates the means of changes in white cell counts in groups of hooded rats after various durations of exposure to nitrous oxide. All the animals also had an incised sutured wound and this and the tail trauma account for the leucocytosis in the controls. Statistical analysis is given in table II.

counts after 1 day's treatment were taken about 2 hours after completion of exposure whereas the results after 2, 3 and 4 days' treatment were obtained 18 to 20 hours after removal of the animals from the ventilation units. In these 2-, 3- and 4-day results an increasing leucocytosis is seen in the controls, and an increasing leucopenia in both the 25 and 60 per cent nitrous oxide treated animals. On analysis, all these trends were significant (table II).

It seemed desirable to recheck the results after 24 hours treatment with nitrous oxide to see if the leucopenia persisted beyond the first 2 hours following the end of exposure. A similar trial was therefore conducted in which repeat counts were not taken until 24 hours after the completion of exposure to nitrous oxide. The 25 per cent nitrous oxide unit was not used on this occasion. A similar leucocytosis was seen in the controls and the animals treated with 60 per cent nitrous oxide; the mean rise of count in the 9 control rats was 4,528 cells/cu.mm, whereas that in the nitrous oxide group of 9 rats was 4,633 cells/cu.mm. It appeared that the leucopenia previously found after 1 day's nitrous oxide treatment was only a temporary effect.

Effects of nitrous oxide in mice.

Unfortunately it was not possible to repeat these investigations with rabbits or guineapigs as these animals appeared too susceptible to the sedative effect of the gas. A similar trial was, however, made with mice. The duration of treatment was 6 days, and 30 mice in three groups of 10 participated in the trial.

The results (table III) revealed a significant depression of total white cell counts both by 25 and 40 per cent nitrous oxide treatment.

Differential white cell counts.

Differential counts were made in rats and mice and changes were seen in the percentage polymorphonuclear count as indicated in table IV.

In rats differential counts made after 1 day's exposure to the atmospheres were similar in the three groups but counts after 3 days showed that a highly significant reduction of the polymorphonuclear percentage accompanied nitrous oxide treatment. The level in rats treated with 25 per cent nitrous oxide was significantly lower than the

TABLE III

Mean changes in white cell counts in mice after 6 days' nitrous oxide.

	Controls (air)			25 per cent nitrous oxide			40 per cent nitrous oxide		
	N	$\bar{x}$	SE	N	$\bar{x}$	SE	N	$\bar{x}$	SE
Change in count per cu.mm	10	−920*	103	10	−2,620†	55	10	−1,900*	77

$\bar{x}$ = mean of changes in white cell counts. N = number of animals in each group.
SE = standard error. * Probability, $P<0.05$. † $P<0.01$.

TABLE IV

Percentage of polymorphonuclear leucocytes after nitrous oxide treatment.

Animal	Duration of treatment (days)	Controls (air)			25 per cent nitrous oxide			60 per cent nitrous oxide		
		N	$\bar{x}$	SE	N	$\bar{x}$	SE	N	$\bar{x}$	SE
Rats	1	6	24.3	2.9	6	22.2	3.3	6	27.3	5.8
Rats	3	12	30.6	2.4	12	15.9	4.2	12	4.3	1.1
Rats	14	10	26.5	3.1	10	8.0	1.4	—	—	—
		Controls (air)			25 per cent nitrous oxide			40 per cent nitrous oxide		
		N	$\bar{x}$	SE	N	$\bar{x}$	SE	N	$\bar{x}$	SE
Mice	6	10	23.2	2.0	10	13.7	2.0	9	8.7	1.8

$\bar{x}$ = mean of percentages of polymorphonuclears. N = number of animals in each group.
SE = standard error.

The polymorphonuclear percentages in rats following exposure to the gases for 1 day are not significantly different in the treated and control animals. With longer durations of exposure in rats and mice the polymorphonuclear percentages are significantly lower in the treated animals ($P<0.01$ in every case).

TABLE V

Percentage of polymorphonuclear leucocytes 3 days after completion of nitrous oxide treatment.

	Controls (air)			25 per cent nitrous oxide			60 per cent nitrous oxide		
	N	$\bar{x}$	SE	N	$\bar{x}$	SE	N	$\bar{x}$	SE
Mean percentage polymorphs (rats)	7	38.3	3.6	7	31.6	3.7	7	14.9	3.2

$\bar{x}$ = mean of percentages of polymorphonuclears. N = number of animals in each group.
SE = standard error.

The animals were treated with the atmospheres for 3 days and differential counts made 3 days later. The counts were taken by cardiac puncture under ether anaesthesia and are therefore not strictly comparable with those in table IV. The percentage of polymorphonuclears is significantly lower in the 60 per cent nitrous oxide group than in the controls ($P<0.001$).
The difference between the 25 per cent group and controls is not significant ($P>0.2$).

controls, while the 60 per cent treated group showed an even greater depression than the 25 per cent group.

Differential counts taken from the rats treated for 14 days with 25 per cent nitrous oxide showed a similar depression of the polymorphonuclears in treated animals when compared with the controls.

The only other component of the differential count showing a change with treatment was the eosinophil count. In rats treated for 3 days with nitrous oxide the mean eosinophil percentage in the controls was 2.0 ± 0.4 per cent and this was significantly higher than that in the 25 per cent (mean 0.8 ± 0.3 per cent) or 60 per cent (mean 0.3 ± 0.1 per cent) nitrous oxide groups of animals.

A depression of the polymorphonuclear percentage was also seen in mice treated for 6 days with nitrous oxide (table IV). The percentages in nitrous oxide treated animals were significantly lower than in the controls.

Delayed recovery of differential count.

In a further series of rats treated for 3 days with nitrous oxide, white cell differential counts were not taken until 3 days after completion of treatment. These showed (table V) that there was still a significant difference between control animals and those treated with 60 per cent nitrous oxide though not between the controls and the 25 per cent nitrous oxide groups.

DISCUSSION

The results of studies in rodents and humans suggest that the effects of long-term nitrous oxide are closely similar in the two cases. Lassen and associates (1956) and Wilson, Martin and Last (1956) first described the granulopenia and leucopenia which was found in patients after about 4 days' exposure to nitrous oxide. This time interval agrees closely with a period of 4 days before significant depression of the white cell counts and altered differential counts were found by Green and Eastwood in their studies in albino rats (1963), and a period of 3 days for similar changes in studies in mice by Yefuni, Federmesser and Smertenko (1964). The latter group stressed the reversibility of the haematological changes and several recent workers (Roth, 1963; Petrovsky and Yefuni, 1965; Pugachev and Kolygin, 1965) regard several days continued sedation and analgesia with nitrous oxide as reasonably safe, provided regular blood checks are made.

While the present studies are in general agreement with those mentioned above they are more indicative of a serious toxicity of the gas, particularly in animals with surgical wounds. Thus a high morbidity and mortality followed 4 days' nitrous oxide treatment in rats with wounds, and the leucopenia appeared more severe than in a similar non-wounded series of animals. A significant leucopenia was present in animals with wounds after the 2nd day of treatment and a marked granulocytopenia was seen in addition after the 3rd day.

A similar prompt fall of leucocyte count was reported by Eastwood and his co-workers in leukaemia patients undergoing nitrous oxide therapy (1963). A review of the reports of leucopenia during nitrous oxide treatment for tetanus (Gormsen, 1955; Lassen et al., 1956; Wilson, Martin and Last, 1956; Last and Nicholas, 1956; Sando and Lawrence, 1958) indicated that the effect was well developed by the 4th or 5th day and that the fall in white cell counts started on the 2nd or 3rd day in some cases. In view of these studies and also the presence of histological changes in the marrow after 48 hours treatment with nitrous oxide in rats (Green and Eastwood, 1963), it appears that even 48 hours exposure to nitrous oxide may be associated with a leucopenic response in humans and a consequent risk of decreased resistance to infection. The depression of white cell count found after 1 day's treatment in these trials was not associated with an altered differential count, and could not be detected when a second 24 hours had elapsed after treatment. One cannot be certain whether this short-term leucopenic effect was due to a redistribution of leucocytes or whether it was an early sign of marrow toxicity.

Recovery from these effects of nitrous oxide has been reported after cessation of treatment (Green, 1964; Yefuni, Federmesser and Smertenko, 1964). It appears possible, however, that recovery may sometimes be delayed a day or two, as an altered differential count was still detected in the present studies several days after a 3-day period of

nitrous oxide treatment, and morbidity and mortality occurred in the 2 days following completion of 4 days' treatment, even though the animals had appeared in satisfactory health during exposure to the gas. A similar delay of over 3 days in recovery from nitrous oxide toxicity was reported by Lassen and associates (1956), by Last and Nicholas (1956), and by Sando and Lawrence (1958) in patients with tetanus; also by Lassen and Kristensen (1959) and Eastwood and associates (1963) after the use of nitrous oxide in patients with leukaemia. This delay in recovery from the leucopenic effects of the gas is in keeping with the toxicity of prolonged intermittent nitrous oxide therapy reported by Ablett (1956) and by Stead, Bush and Roth (1962).

If continuous nitrous oxide therapy is considered essential then regular white cell counts are advisable. Nevertheless, the presence of normal total white cell counts will not necessarily indicate absence of a nitrous oxide effect as it was shown in the first trial that long-term therapy with 25 per cent nitrous oxide could inhibit a leucocytosis which was seen in the control series in response to trauma.

Mechanism of nitrous oxide toxicity.

The mechanism of nitrous oxide toxicity is not fully understood. Studies of the bone marrow revealed a progressive hypoplasia (Green and Eastwood, 1963). The reported toxic effects of nitrous oxide on mitosis in embryonic myoblasts (Kieler, 1957) together with the decreased radiosensitivity of tissues caused by nitrous oxide (Evans and Orkin, 1962) both suggest that the gas may cause leucopenia by selectively inhibiting mitosis in some of the blood-forming elements in the marrow. Yefuni, Federmesser and Smertenko (1964), however, found no changes in chromosome structure in mice marrow cells after nitrous oxide treatment.

Higher oxides of nitrogen, nitric oxide and nitrogen dioxide, are possible contaminants of nitrous oxide. Leucopenia and granulocytopenia were reported in industrial workers exposed to these oxides in the form of "nitrous fumes" for prolonged periods (Pfenninger, 1943), and Vigdortschik and associates (1937) also regarded leucopenia and granulocytopenia as probable symptoms of mild chronic poisoning with nitrogen oxides. A leucopenic effect of higher oxides of nitrogen is more understandable in view of their marked chemical activity than in the case of the chemically inert nitrous oxide. Consequently the presence of higher oxides in nitrous oxide is of considerable importance. The present accepted standards for intermittent exposure of industrial workers to nitric oxides during working hours are 10 p.p.m. (v/v) in Britain and 5 p.p.m. (v/v) in the U.S.A. In the U.S.S.R. the safety limit is set at a lower level (Riasanov, 1959). In the currently described studies the standard of purity of nitrous oxide was accepted as under 1 p.p.m. of higher oxides (A. Bracken, personal communication). Green and Eastwood reported the purity of their nitrous oxide as better than 10 p.p.m. of higher oxides. The exact level (in p.p.m.) at which the leucopenic effect of nitric oxides occurs is of added interest as appreciable quantities of the oxides are absorbed by smokers (Bokhoven and Niessen, 1961), and emitted in the exhausts of diesel vehicles (Gerhardson, 1963). In addition to the risk of contamination of nitrous oxide with higher oxides there is a possibility of traces of nitrous oxide being metabolized to chemically more active oxides in the marrow cells.

Pfenninger (1943) also described methaemoglobinaemia in her patients suffering from "nitrous fumes". In the present studies, however, no evidence of methaemoglobinaemia was found in the blood of 14 rats treated with nitrous oxide for 3 days, the blood being examined under the reversion spectroscope.

An alternative theory of the mechanism of nitrous oxide toxicity is that there may be a link between drugs producing anaesthesia and effects on mitosis and leucocytes. Thus the gases, nitrogen, xenon and argon, when used under hyperbaric conditions, are both anaesthetic and have analogous effects to those of nitrous oxide in reducing oxygen sensitivity of tissues to radiation (Ebert, Hornsey and Howard, 1958; Ebert and Hornsey, 1958). Pentobarbitone, too, has been shown to have a similar action (Evans and Orkin, 1962). Many anaesthetic agents other than nitrous oxide have also been shown to be toxic to leucocytes in patients (Tempo, 1965) and to have a leucopenic effect in animals (Smith et al., 1948; Graca and Gärst, 1957; Usenik and Cronkite, 1965).

CONCLUSIONS

From the present studies and a review of the literature it is doubtful whether continuous nitrous oxide therapy can be recommended for routine postoperative sedation for periods of over 48 hours. There appears to be a risk that the nitrous oxide may inhibit a leucocytosis, in response to trauma and infection, and may lower patients' resistance. The results should not necessarily be taken as indicating any toxicity for intermittent short-term administration of the gas in the course, for instance, of postoperative physiotherapy (Parbrook, 1965), in general anaesthesia, or for pain relief in labour.

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EFFETS LEUCOPENIQUES D'UN TRAITEMENT PROLONGE PAR LE PROTOXYDE D'AZOTE

SOMMAIRE

Chez des rongeurs avec ou sans plaies chirurgicales on a étudié les effets leucopéniques du protoxyde d'azote. Les animaux ont été exposés à l'une des atmosphères suivantes: (1) air; (2) 25 pour cent de protoxyde d'azote; (3) 60 pour cent de protoxyde d'azote (rats), 40 pour cent de protoxyde d'azote (souris); le pourcentage d'oxygène restant maintenu dans tous les cas à 21 pour cent. Un effet leucopénique et la suppression de la leucocytose posttraumitique normale ont été observés avec toutes les concentrations de protoxyde d'azote après deux jours de traitement. L'effet du protoxyde d'azote à 60 pour cent a été plus fort que celui du protoxyde d'azote à 25 pour cent après sept jours, mais la différence était moins nette au cours des quatre premiers jours. L'effet leucopénique était plus prononcé chez les animaux qui étaient atteints par des plaies chirurgicales. La granulopénie survint au troisième jour, mais non pas après un seul jour de traitement par le protoxyde d'azote, et persiste pendant trois jours au moins après la fin du traitement. La sécurité du traitement par le protoxyde d'azote et les différents mécanismes possibles pour l'explication de la toxicité sont discutés.

LEUKOPENISCHE WIRKUNGEN EINER AUSGEDEHNTEN LACHGASBEHANDLUNG

ZUSAMMENFASSUNG

Bei Nagetieren mit und ohne Operationswunden wurden Untersuchungen der leukopenischen Effekte von Lachgas durchgeführt. Die Tiere wurden einem der folgenden Gasgemische ausgesetzt: (1) Luft; (2) 25prozentigem Lachgas; (3) 60prozentigem (Ratten) oder 40prozentigem (Mäuse) Lachgas, wobei die Sauerstoffkonzentration in jedem Fall bei 21 Prozent gehalten wurde. Nach zweitägiger wurde bei allen Lachgaskonzentrationen eine leukopenische Wirkung sowie eine Unterdrückung der normalen Leukozytose nach Traumen beobachtet. Nach sieben Tagen war der Effekt von 60prozentigem Lachgas stärker als der von 25prozentigem Lachgas, doch der Unterschied war in den ersten vier Tagen weniger deutlich. Die leukopenische Wirkung war bei den Tieren mit Operationswunden stärker. Eine Granulozytopenie trat nach drei Tagen, nicht jedoch schon nach eintägiger Lachgasverabreichung auf und hielt wenigstens drei Tage nach Beendigung der Behandlung an. Die Sicherheit der Lachgasbehandlung und deren mögliche Toxizitätsmechanismen werden diskutiert.

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TOXICITY OF NITROUS OXIDE

Dr. G. D. Parbrook

ALTHOUGH NITROUS OXIDE is one of the safest anaesthetics, with minimal effects on the metabolism, there are several potential toxic effects associated with its clinical use.

Its long-term toxicity to the marrow is well known, but the safe duration of continuous therapy is less than originally envisaged and the effects on immunity response and on the leucocyte response to trauma may be present within two days of continuous nitrous oxide.

Hypotension has been reported when a nitrous oxide/oxygen mixture has been administered to patients receiving halothane/oxygen anaesthesia. This toxicity has not been confirmed by other workers who show that, in equipotent levels, nitrous oxide/oxygen/halothane anaesthesia gives less hypotension than halothane/oxygen.

Too low a dose of nitrous oxide has its own toxicity with risks of awareness during procedures. Failure to use an adequate level could be due to machine failure and a different apparatus failure can give an even more serious risk—that of rapid hypoxia from ventilation with pure nitrous oxide. To prevent this, modern dental anaesthetic machines

incorporate safety cut-outs on the nitrous oxide supply, working on oxygen pressure, but these safety devices are still absent from most other anaesthetic machines.

There has been recent controversy on the importance of post-operative diffusion hypoxia as the fall of oxygen tension gives little effect on the oxygen saturation in the blood of fit healthy patients. In practice, diffusion hypoxia remains a real entity in the more ill patients and those with bronchitis as they will already be functioning on the steep slope of the oxygen dissociation curve with risks of serious falls of oxygen saturation. Other diffusion toxic effects due to diffusion of nitrous oxide into pockets of air in the body (inner ear, bowel, pneumothorax, etc.) are well known.

Although nitrous oxide has mild teratogenic effects in some experimental circumstances, it is unproven whether any effect is present in humans. It would be difficult to protect a patient from such effects as the real danger from any teratogenic agent is in the first few weeks of pregnancy, before a patient realizes she is pregnant.

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General biological effects of the helium-xenon series of elements

H. R. SCHREINER
Union Carbide Corporation, Linde Division
Research Laboratory, Tonawanda, New York

MY PURPOSE is not to convince you that the so-called "inert" gases are capable of showing biological activity—this will soon become self-evident—but to demonstrate that this biological activity may be observed on levels of organization ranging from man to enzymatically active protein molecules. In doing this, I will try to separate the truly pharmacological effects of these gases from the biological responses which they may elicit by modifying the physical properties of the gaseous environment. Finally, I will engage in some speculation concerning the molecular mechanism by which the elements of the helium-xenon series are capable of exerting pharmacological activity.

Gases that are not metabolized by the mammalian body, such as nitrogen and the gases of the helium group of the periodic system of elements, i.e., helium, neon, argon, krypton, and xenon, have long been regarded as pharmacologically inactive. Even though divers have known for more than 100 years that the breathing of compressed air can produce narcotic effects (46), the scientific community has been remarkably slow in recognizing that nitrogen is by no means unique in this respect. The initial steps toward recognition of the latent pharmacological activity of helium-group gases were those taken by Lawrence (52) and Lazarev (53) who produced for the first time experimental evidence that the breathing of xenon at partial pressures of less than 1 atm can produce depressive effects in animals and in man. However, it remained for Cullen and his associates (27, 29) at the University of Iowa to demonstrate unequivocally the anesthetic potency of xenon in man. This event marks the beginning of the systematic study of the biological effects of helium group gases which has opened new avenues in the search for mechanisms of anesthesia (37, 60, 69, 94) and, in addition, is providing us today with much of the biological information that is required for the intelligent selection of artificial gaseous atmospheres for manned spacecrafts (2, 7, 30, 35, 42, 48, 73, 74, 77, 96, 101, 107, 109) and for undersea habitats (8, 47, 49, 57, 90, 103). The need to protect man and to permit him to remain fully functional as he ventures out into space and into the inner reaches of the ocean lends new and perhaps unexpected significance to the subject matter of this Symposium. It therefore seems particularly appropriate to acknowledge the significance of the pioneering work of Cullen, Pittinger, Featherstone, and their associates who, by their early concern with the mechanism of xenon anesthesia (39, 61, 65–71), have laid the foundations of inert gas physiology and pharmacology.

I think that the first question one should ask in considering the physiological significance of so-called "inert" gases is whether or not their total absence leads to measurable physiological alterations.

We know that the removal of nonmetabolized gases from the respiratory environment does not lead to immediate deleterious effects in small animals (56) and information available to date from the U. S. manned spaceflight program indicates that for limited periods of time at least, man can maintain his physiological functions within normal limits in the absence of so-called inert gases. While it is therefore quite likely that helium-group gases are not essential to life (11), we still cannot exclude with certainty the possibility that rigorous deprivation of an animal organism of a suitable nonmetabolizable inert gas, such as nitrogen, may result in the eventual development of physiological abnormalities (10, 11, 76, 78, 95, 97, 99, 100, 104).

It is important to realize that any modification in atmospheric composition or pressure is by necessity associated with changes in one or more of the physical bulk properties of the gaseous environment, such as viscosity, density, or thermal conductivity, to name a few. Changes in these environmental parameters may elicit physiological responses that do not depend on the ability of the gas to interact physically with functional elements of the living cell but that depend rather on the contribution made by the gas molecules to the physical nature of the atmosphere. A case in point is the distortion of the human voice by helium (4, 25, 26, 34, 92, 98). Another is the effect of gas density on the work of breathing (18, 43, 47, 51, 55, 62, 102). Yet a third is the metabolic stimulation of mammals by exposure to gases

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with high thermal conductivities such as helium (9, 12, 22, 24, 40, 41, 50, 54, 106).

Our own work on the metabolic effects of helium-group gases on small mammals has produced evidence (Fig. 1) for the importance of the thermal properties of the gaseous environment in determining the level of resting oxygen consumption (48). These experiments were conducted at a Po_2 of 180 mm Hg on rabbits and rat exposed to oxygen and to mixtures of oxygen and helium, neon, nitrogen, or argon at 755 or 474 mm Hg (the small symbols in this figure refer to experiments at simulated altitude). The average rate of oxygen consumption over a 7-day period at a temperature of 25–26 C correlates quite well with the relative rate of convective heat transfer H_2/H_1 from the animals into the various gaseous environments, and is clearly not affected by properties that are peculiar only to the helium-group gases employed in this study.

Earlier studies by Cook and South (22, 23, 93, 94) had indicated that helium is capable of affecting the in vitro rate of respiration of tissue slices, a phenomenon for which a thermal mechanism cannot be invoked. However, recent studies by Rodgers (75) have shown that helium does not influence the in vitro oxygen consumption of rat tissues at ambient and slightly elevated pressures. The discrepancy between these results and those of South and Cook may be related to the observation that small differences in Po_2 in the range from 0.20 to 0.26 atm can produce relatively large differences in oxygen consumption, probably because of diffusion limitations.

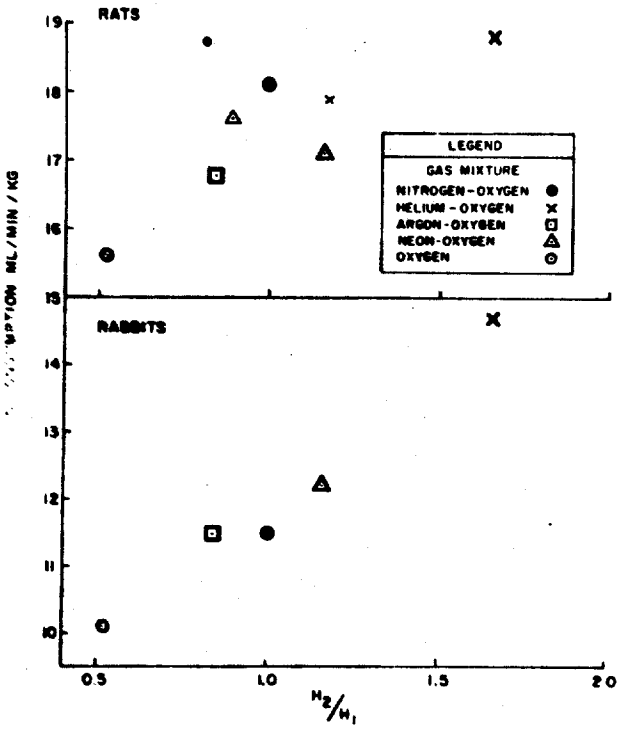

FIG. 1. Effect of forced convective heat loss on oxygen consumption. H_2/H_1 represents heat loss relative to air at 1 atm as calculated by Epperson's equation (35). For experimental conditions, see text.

This issue is further compounded by recent observations made by Maio and Neville (58, 59) of non-specific reductions in the consumption of oxygen by yeast and rat liver slices in the presence of helium, nitrogen, or argon. Since the oxygen consumption of tissue homogenates is not depressed by inert gases, these authors conclude that inert gases may act on a membrane mechanism for oxygen transport into the cell.

This controversy not withstanding, helium-group gases are in fact capable of producing distinct pharmacologic effects of which xenon anesthesia is but one example. In fact, all so-called inert gases are known today to produce measurable effects in biological systems if their concentration is sufficiently high (72). These effects presumably have their origin in molecular interactions about which we are to hear more. It should suffice for the moment to point out that these interactions depend on certain molecular properties that lend pharmacologic significance to helium-group gases in particular and to all so-called inert gases in general. I should like now to consider this aspect of helium-group gases.

By all criteria that are commonly used to gauge the narcotic potency of a gas, helium appears to be the least effective member of its group. Indeed, even at a partial pressure as high as 20 atm, no performance decrement could be reliably determined in men exposed to helium for 48 hr (49, 57). That helium is not entirely devoid of pharmacologic potency, however, may be inferred from the results of recent studies by Brauer and his colleagues. In a quantitative evaluation of the depressive effect of mixtures of helium and nitrogen on mice and rhesus monkeys, these workers found that as the proportion of helium in their compressed gas mixtures increased, the observed effect became greater than the effect of nitrogen alone. On the assumption that the narcotic actions of helium and nitrogen are qualitatively identical and add linearity, it is possible to extrapolate to a relative depressive potency of helium which comes close to 20% [illegible] of nitrogen.

A considerable difference in the [illegible] concentration of helium was noted in these experiments: rhesus monkeys convulsed when helium pressures reached 45–47 atm (14) while convulsions in mice were seen only when the helium pressure was increased to 75–100 atm (15). This apparent species difference in the threshold pressures of response to helium is also seen for xenon: anesthesia is produced by 0.8 atm of xenon in man only (36), for the rat (44), rabbit (43), dog (33), and monkey (66), xenon pressures in excess of 1 atm are required to reach an anesthetic end point.

No threshold pressure for a pharmacologic effect of neon on man has been determined to date. In our own studies (90) we were unable to detect a performance decrement during a 30-min exposure to 10 atm of this gas (Fig. 2). Argon has been described as showing greater narcotic effectiveness than nitrogen (3), and krypton is regarded as less narcotic than xenon (17). We can then

fore arrange the helium-group gases and nitrogen in the following order of increasing narcotic potency in man:

$$He < Ne < N_2 < Ar < Kr < Xe$$

This relative order is further substantiated by a number of in vivo and in vitro studies of central nervous system activity and nerve impulse conduction in animals as a function of the nature and concentration of helium-group gases (5, 6, 19–21, 45).

This order of effectiveness has been noted with few exceptions in every systematic investigation of the biological effects of helium-group gases that has been undertaken to date (31, 91). It is especially clearly seen in the results of some of our early studies on the filamentous fungus *Neurospora crassa* (89). At a total pressure of 1 atm there exists a striking dependence of the linear growth rate of this organism on the nature of the oxygen diluent (Fig. 3). This dependence can be expressed as a linear relationship to the square root of the molecular weight. However, since other gases such as sulfur hexafluoride (84) and nitrous oxide (88) affect this system in a manner that cannot be correlated with molecular mass, the linearity of this relationship is not general and probably fortuitous for helium-group gases.

In searching for the mechanism of this effect, we discovered a striking and reversible inhibition by krypton, and by xenon in particular, of the rate of protoplasmic streaming in the hyphae of this mold (86). Figure 4 shows a time record of the relative protoplasmic streaming velocity in *Neurospora crassa* exposed alternately to xenon and nitrogen at a constant partial pressure of oxygen. Qualitatively identical observations have been recorded for the replacement of helium by

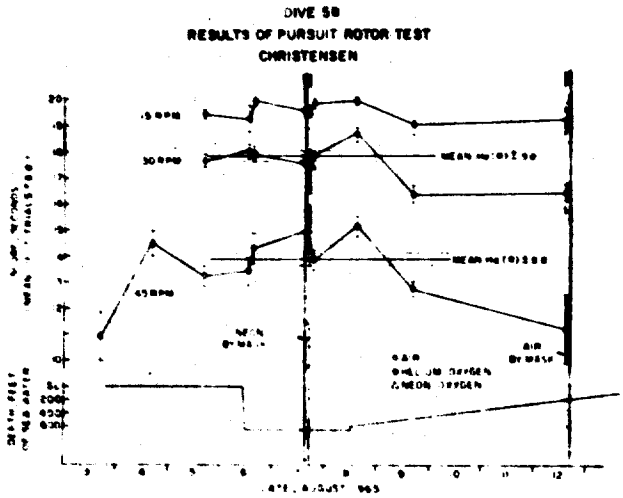

FIG. 2. Effect of compressed inert gases on the performance of a simple motor task (rotary pursuit apparatus) requiring eye-hand coordination. Average time of contact in seven 20-sec trials is shown for three tunnable speeds while breathing oxygen-inert gas mixtures over a 9-day period. The pressure history during the experimental exposure is indicated in the bottom portion of this figure. 33 ft of seawater = 1.00 atm.

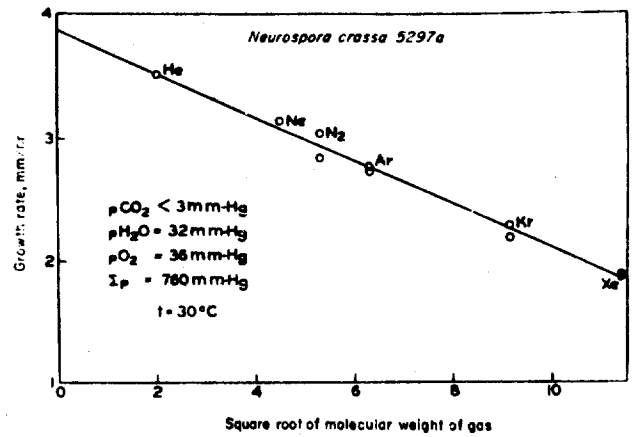

FIG. 3. Mycelial growth response of *Neurospora crassa* 5297a on semisolid medium to the presence of gases of the helium-xenon series of elements. (From Schreiner et al. (89). Copyright 1962 by the American Association for the Advancement of Science).

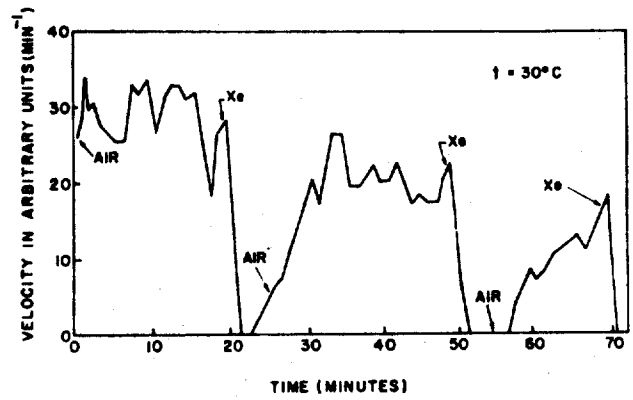

FIG. 4. Effect of 80% xenon-20% oxygen on protoplasmic streaming velocity of *Neurospora crassa* 5297a as determined by measuring the time required for individual organelles to traverse the length of two adjacent hyphal cells selected for microscopic observation.

xenon. It is interesting to note that, although protoplasmic streaming comes to a complete halt on contact with xenon, it tends to become reestablished at a lower rate within 30–60 min. From a theoretical point of view, it is interesting to speculate that this may be an adaptive phenomenon.

Gas density does not account for the effect on *Neurospora crassa* of gases of the helium group. This is clearly demonstrated by the growth responses shown in Figs. 5 and 6 of the mold to these gases at pressures ranging to 120 atm (17). Partial pressures required for 50% inhibition of growth were: Xe (0.8 atm), Kr (1.6 atm), Ar (3.8 atm), Ne (35 atm), and He (~300 atm). Related studies with mammalian cells (16) showed very little significant effect of helium-group gases at a partial pressure of 0.75 atm on 3-day growth of monolayer cultures of HeLa cells (Fig. 7). At elevated pressures, however, differences in growth response to xenon, krypton, and argon can be clearly discerned (Fig. 8). Recent extension of this work

to pressures of up to 200 atm is permitting us now to resolve differences in the biological effectiveness of helium, neon, and nitrogen in this system.

There may exist more than one single mechanism by which helium-group gases exert cellular effects. Within a cell the dissolved gas could make its presence felt through the nonspecific occupation of space in a local region involved in critical physical or biochemical processes. In addition, the dissolved gas molecule may specifically

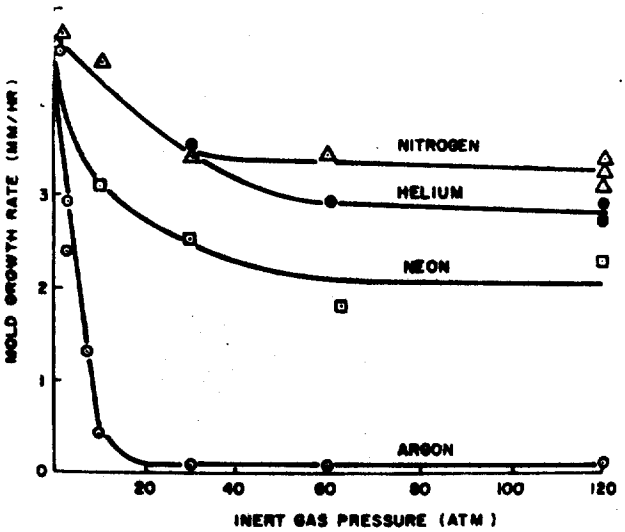

FIG. 5. Relation between partial pressures of nitrogen, helium, neon, and argon and the mycelial rate of growth of *Neurospora crassa* 5297a on semisolid medium. Partial pressure of oxygen: 0.2 atm. (From Buchheit et al. (17).)

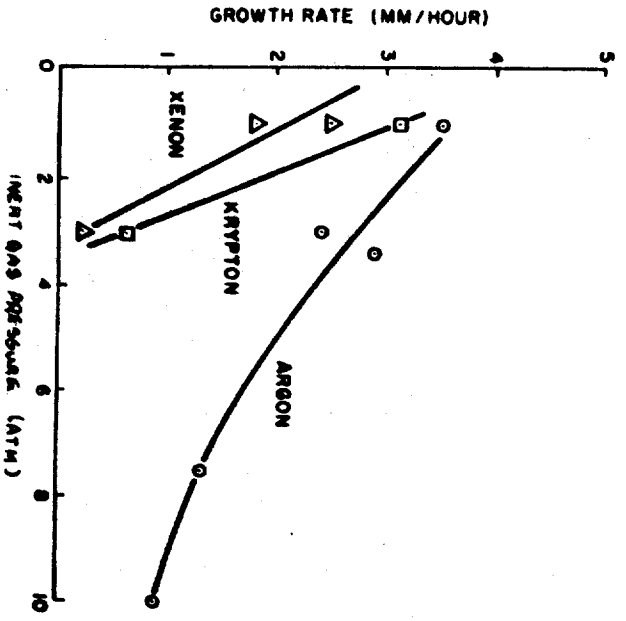

FIG. 6. Relation between partial pressures of argon, krypton, and xenon and the mycelial rate of growth of *Neurospora crassa* 5297a on semisolid medium. Partial pressure of oxygen: 0.2 atm. (From Buchheit et al. (17).)

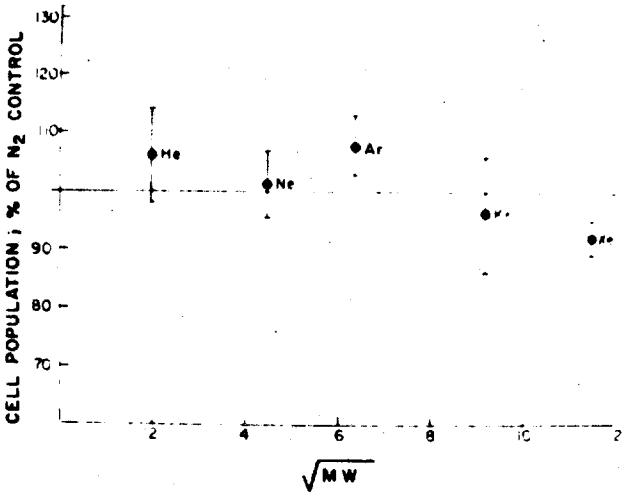

FIG. 7. Monolayer growth response of HeLa cells to members of the helium-xenon series of elements at a partial pressure of 0.75 atm. P_{O_2} = 0.20 atm, P_{CO_2} = 0.05 atm. Each point ± 1 standard deviation represents the mean number of attached cells after 4 days of exposure as determined in three or four experiments, five replicates per experiment. Average cell population in the nitrogen control experiment increased from 1×10^5 to 5.8×10^5 cells/ml over a 3-day period. Differences in cell populations between test cultures and nitrogen control cultures are not statistically significant. Difference in growth responses to helium and xenon is significant at $P < 0.01$. (From Fig. 1, Bruemmer, J. H., B. B. Brunetti and H. R. Schreiner, *J. Cell. Physiol.* 69: 89, 1967.)

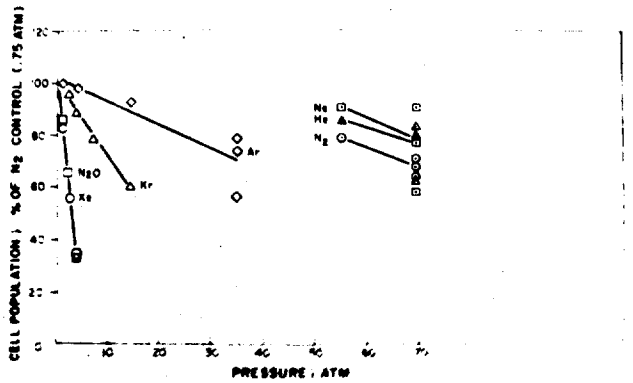

FIG. 8. Monolayer growth response of HeLa cells to members of the helium-xenon series of elements as a function of total pressure. P_{O_2} = 0.2 atm, P_{CO_2} = 0.05 atm. (From Fig. 2, Bruemmer, J. H., B. B. Brunetti and H. R. Schreiner, *J. Cell. Physiol.* 69: 337, 1967.)

compete by adsorption at an interface with some essential cellular constituent. In such postulated modes of involvement, specific long-range induction and dispersion interactions would be of critical importance. Since the stability of the products of such intermolecular interactions is to a large measure proportional to the molecular polarizability α of the gas molecules (85), there should be—and to a considerable extent there is—a positive correlation between molecular polarizability and anesthetic potency of gases (105). We have found a linear relationship between molecular polarizability and the partial pressures of helium-group gases required for the

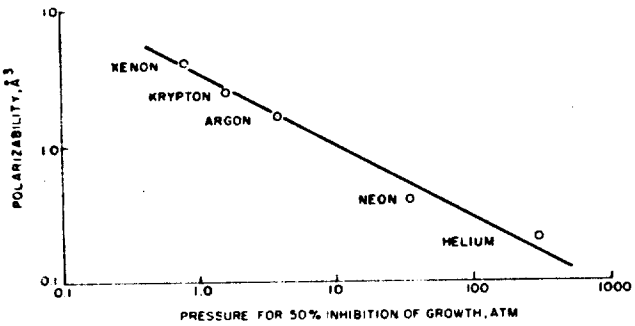

FIG. 9 Molecular polarizabilities of members of the helium-xenon series of elements and partial pressures of these gases required for the 50% inhibition of growth of *Neurospora crassa* 5297a. From Buchheit et al. (17).

TABLE 1. *Activity of tyrosinase in the presence of inert gases superimposed at 30.6 atm on 1 atm air*

Number of Observations	Superimposed Gas	Relative Activity	Statistical Significance at $P = 0.005$ Versus				
			Control	Helium	Neon	Argon	Nitrogen
12	None	100					
5	Helium	84	Yes				
7	Neon	70	Yes	No			
5	Argon	68	Yes	No	No		
5	Nitrogen	60	Yes	Yes	No	No	
6	Nitrous oxide	37	Yes	Yes	Yes	Yes	Yes

50% inhibition of *Neurospora crassa* growth (Fig. 9). A similar relationship can also be demonstrated for our still incomplete data on the growth inhibition of cultured human cells.

Intact cellular structure and functionality is by no means a prerequisite for the demonstration of biological effects of inert gases. We have observed effects of these gases on the activity of purified enzyme systems, including the oxygenases lipoxidase (lipoxygenase 1.99.2.1 IUB) and tyrosinase (catecholoxidase 1.10.3.1 IUB) (87) and the hydrolases α-chymotrypsin, lencine amino peptidase.

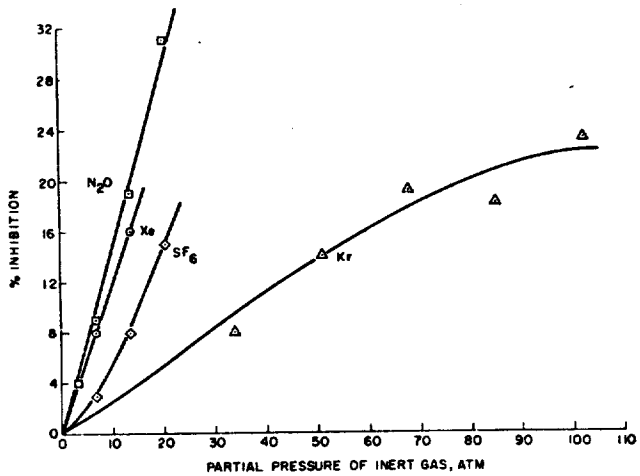

FIG. 10. Inhibition of acetylcholin esterase activity by nitrous oxide, xenon, sulfur hexafluoride, and krypton as a function of partial pressure of these gases. P_{O_2} = 0.2 atm, P_{N_2} = 0.8 atm.

and acetylcholinesterase (32). The strength of these effects varies considerably between different enzymes, indicating relatively large quantitative differences in susceptibility of purified enzymes to inhibition by inert gases. Typical examples of the degree of inhibition that has been obtained are shown in Table 1 for tyrosinase at a constant partial pressure of 30.6 atm, and in Fig. 10 for acetylcholinesterase at pressures ranging to over 100 atm. Since the association of proteins with nonmetabolizable gases is now well documented (38, 79–83), it is reasonable to postulate that the observed changes in enzymic activity may be the result of structural alterations in the enzyme molecules brought about by interaction with helium-group and other inert gases.

In summary, then, it is possible to conclude that the helium-group gases are *1)* not essential to life, *2)* affect physiological processes by modifying physical properties of the gaseous environment, and *3)* produce pharmacologic effects by modifying cellular functions through interactions on the molecular level. It is this last aspect of helium-group gases which will be dealt with by papers following this.

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26

Thomas H. Shepard, M.D., and B. Raymond Fink, M.D.

Teratogenic Activity of Nitrous Oxide in Rats*

After Lassen demonstrated the toxic potential of nitrous oxide to human bone marrow (1), the possibility of harm to other tissues with rapid cell growth, such as the embryo, began to arouse concern. Eastwood and Rector (2) and Smith and Moya (3), whose work with chick eggs is reviewed in more detail in Chapter 25, found an appreciable incidence of embryonic death after exposure to nitrous oxide but only a few obvious malformations in the survivors. Parbrook, Mobbs and Mackenzie (4) began with the rat, noting that pregnant does, kept in 60% nitrous oxide for 48 hr during the first 8 days after mating, gave birth to few live offspring. The condition of the latter was not described. These authors then went to chick embryo explants (5) but detected no adverse effect on development after 24-hr exposures to nitrous oxide.

We took up the problem in the rat, partly because its bone marrow was already known to be as susceptible to nitrous oxide as that of man, and partly because the study of the effect in a mammalian species was of intrinsic interest. Our preliminary results with 2- to 6-day exposures to nitrous oxide have been previously reported (6). This paper will review those initial studies as well as the consequences of 24-hr exposures.

METHODS

Virgin Sprague-Dawley rats giving the characteristic estrus response (7) were placed with a male at about midnight. Pregnancy was assumed if a copulatory plug and vaginal spermatozoa were present the next morning. This day was designated as day 0 in order to make the day of gestation numerically the same as the age in days of the embryo. The pregnant animals were maintained on Purina lab chow and given water

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ad libitum. Starved animals received no food, but their supply of water was unlimited.

During the period of treatment, the animals were placed in an air-tight, transparent box singly or in pairs and the gas mixture was flowed through the box at a rate of 2 liters per min. The box was provided with a wire-mesh floor under which was spread a layer of soda-lime carbon dioxide absorbent. The composition of the box atmosphere was determined frequently by infrared nitrous oxide and carbon dioxide and paramagnetic oxygen gas analyzers. The carbon dioxide concentration did not exceed 0.3% and generally was below 0.1%. The temperature in the box was about 3°C above room temperature and generally at 23 to 26°C.

During the preliminary study, in which the exposure periods were longer than 1 day, the gas mixture consisted of 45 to 50% nitrous oxide, 21 to 25% oxygen and nitrogen for the experimental animals, and either air from a compressed air cylinder or a mixture of 21% tank oxygen and 79% nitrogen in the case of the controls. There were no detectable differences between the results with the two types of controls.

The gas mixture used for 1-day exposures consisted of 70% nitrous oxide and 30% oxygen. Three groups of control animals untreated with nitrous oxide used in this experiment were: 1) starved in air, 2) exposed to 30% oxygen and 70% nitrogen and 3) exposed to 10% oxygen and 90% nitrogen.

After treatment the animals were returned to a standard cage until day 20, when they were killed by cervical dislocation. The fetuses were weighed upon removal, the crown-rump length was measured and the sex was determined by perineal inspection and, on occasion, by laparotomy and inspection of the gonads. The number of corpora lutea of pregnancy was counted, and was usually found to almost coincide with the total number of implantations plus resorptions. After the preliminary stages of the investigation, most of the litters were fixed in 95% ethanol preparatory to digestion with potassium hydroxide solution and staining of the skeleton with alizarin red S, but a few were fixed in alcoholic Lillie's solution and subsequently sliced with a razor blade and inspected for anomalies of the brain and viscera.

RESULTS

Exposure for 2 to 6 Days to 50% Nitrous Oxide (6)

The commonest disturbances produced by nitrous oxide when treatment commenced on day 8 were 1) death and resorption of embryos and 2) abnormalities of the vertebrae and ribs. The percentage of survivors and average fetal weight decreased with duration of exposure to nitrous oxide (table 26.1). The experimental litters comprised 105 live fetuses,

TABLE 26.1

Weight, length, sex ratio and incidence of malformations in rat fetuses from mothers placed in 50% N_2O and 21% O_2-N_2 on day 8 of pregnancy, and from 21% O_2-N_2 controls

Days in 50% N_2O	Implantations	Resorptions	Survivors	Average weight	Average length	Sex ratio*	Survivors with skeletal malformation	Other malformation
				g	mm			
0 (Control)	77 (6)†	1	76	3.7 ± 0.1‡	36.5 ± 0.2‡	39:37	2 (46)§	0 (29)§
2	36 (3)	7 (19%)	29 (81%)	3.2	34	11:18	15 (15)	0 (14)
4	88 (7)	22 (25%)	66 (75%)	2.8	31	22:44	42 (42)	4 (23)‖
6	23 (2)	13 (57%)	10 (43%)	2.7	33	2:8	(0)	0 (10)
Experimental totals	147 (12)	42 (28%)	105 (72%)	2.9 ± 0.1	31.8 ± 0.3	35:70	57 (57)	4 (47)

* Ration of male:female.
† The number of litters is in parentheses.
‡ Results given ± standard error.
§ The number of fetuses examined is in parentheses.
‖ Excluding one dwarf preserved intact for photography.

of which 57 were stained with alizarin; every one of the latter exhibited defects of vertebral ossification. Fifteen of the 105 were dwarfs—fetuses with a crown-rump length of 2.5 cm or less (normal crown-rump length, 3.7 cm). Other common types of skeletal abnormalities encountered were partial or total absence of ossification of one or more ribs and partial fusion of ribs, often associated with irregular development of the centra of adjacent vertebral bodies (fig. 26.1). Centers of ossification were absent on one side in some vertebrae. There were many instances of fusion between ossification centers in adjacent vertebrae. With two exceptions, all of the controls had normally developed skeletons with full complement of vertebral and costal ossification centers. The exceptions had supernumerary rib rudiments. Of the 47 experimental fetuses sectioned, four were found to have major visceral malformations, including one with hydronephrosis, one with cardiomegaly and three with hydrocephaly (fig. 26.2). In the small group of fetuses exposed for 6 days, skeletal clearing was not performed, which perhaps accounts for a low incidence of malformations observed (table 26.1). The average weight of the fetuses in the nitrous oxide treated groups was 2.9 ± 0.1 g (S.E.) as against 3.7 ± 0.1 g in the control group. The crown-rump length averaged 31.8 ± 0.3 mm in the treated, as compared to 36.5 ± 0.2 mm in the control. Applying the single tailed t test, the differences between these means are highly significant ($p < 0.001$). Shortening of the spine was apparently not accompanied by corresponding shortening of the limbs, since the length of the ulna in the cleared skeletons did not differ significantly in the treated and control groups. The placental weight/fetal weight ratio did not differ significantly in the two groups.

Figure 26.1. Alizarin stained skeletons of day 20 rat fetuses. Left: control. Middle and right: representative examples of skeletal anomalies after exposure to 50% nitrous oxide on days 8 to 11 of pregnancy. The middle specimen was a dwarf. Magnification uniform; scale in millimeters. Reproduced through the courtesy of *Nature* (6).

The sex ratio among the surviving fetuses of the nitrous oxide treated group was 35 males to 70 females, whereas in the controls the ratio was 39 males to 37 females. We believe the difference in the two ratios to be significant ($\chi^2 = 5.2$; $p = 0.023$). It is noteworthy that of the 147 implantation sites in the nitrous oxide exposed group, 42 were resorptions, as against only one resorption in the 77 controls. In the 2- and 4-day treated group the number of surviving females exactly equalled the sum of the surviving males and resorptions. The presumption is that the resorptions were mostly males and that the lethal effect of the treatment on embryos apparently occurred selectively on those of the male sex. In the 6-day group there were more resorptions than survivors, suggesting that in this group some of the female embryos had died also. A selective mortality of this degree in the male has not been observed previously in experimental models but is known to occur in the

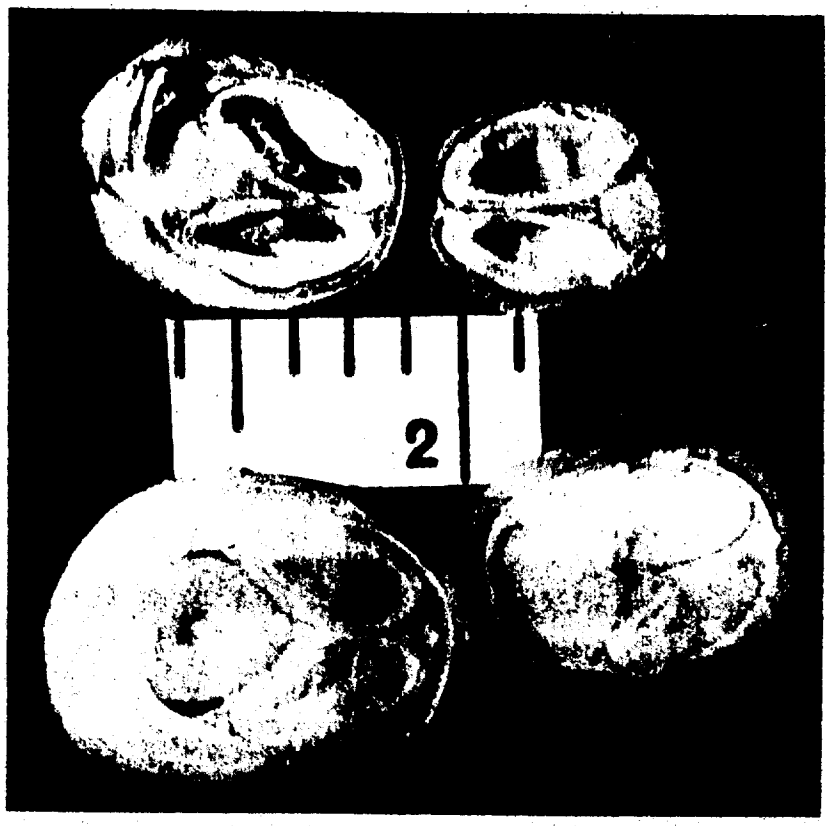

Figure 26.2. Hydrocephalus in a fetus from a rat treated from day 8 through day 12 of pregnancy. At the top is a razor cross section to show dilatation of the cerebral ventricles with thinning of the cortex; at the bottom is a control cross section from a normal fetus. Scale in 1/8 inch.

human fetus during early pregnancy. Whether this effect is mediated by direct gene action or by sex hormones produced in the fetal gonad cannot be decided on the present evidence, although it should be noted that most of the embryos in this study were treated before differentiation of the gonad.

Exposure for a Single Day to 70% Nitrous Oxide

To find the time of embryonic development most sensitive to nitrous oxide exposure, the does were exposed for 24-hr periods on day 5 through day 11. These studies were a necessary preliminary step to forming more specific hypotheses about the mechanism of teratogenic action of nitrous oxide. Furthermore, 70% nitrous oxide more closely approaches concentrations used in humans. The oxygen concentration was increased to 30% in an attempt to preclude undetected mild hypoxia in the mother. Examination of arterial blood in parallel experiments, to be reported elsewhere, showed that hypoxia was indeed avoided.

The results of 1-day exposure to nitrous oxide, presented in table 26.2 and figures 26.3 through 26.8, indicate that a peak incidence of malformation occurred after exposure on day 9 (fig. 26.3). The axial skeletal abnormalities were similar to but of less marked degree than those described above in animals treated for more than 1 day. Treatment on day 9 resulted in 54 of 108 survivors with one or more abnormalities. Of this group of 54, 51 had completely separated vertebral centra, 15 had unilateral absence of an ossified centra with scoleosis; 12 had a shortened or absent rib and 5 had fusion of ribs (fig. 6 and 7). A predilection for vertebrae T-10 through L-1 was observed (fig. 26.4). Although several litters were examined using the razor blade technique, no abnormalities

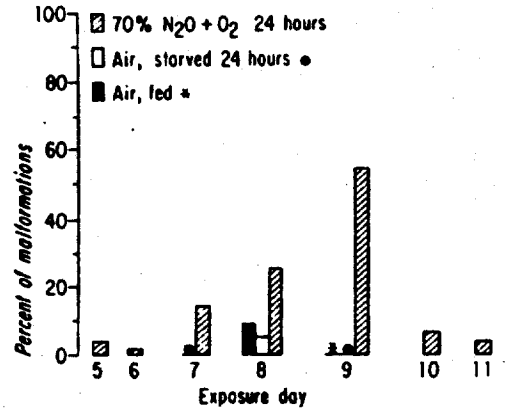

Figure 26.3. The percentage of 20-day survivors with vertebral malformations following exposure to N_2O on days 5 through 11. The malformations included complete separation or absence of vertebral ossification centra. See table 26.2.

of internal organs were detected. One dwarf was found in each of the groups treated on day 7, 8 and 11; a hemangioma was found in one of the animals treated on day 9. In the starvation controls, one dwarf and one edematous fetus were discovered, and in the hypoxia group there was one dwarf. No significant increase in resorptions was found and no increase over normal of unaccounted corpora lutea occurred. The total number of survivors (608) and resorptions (25) in the nitrous oxide treated group was only three less than the number of corpora lutea of pregnancy (636). The ovarian bodies recognized as corpora lutea of pregnancy were rounded and engorged, and those less than 2 mm in diameter were not counted. The average weights of the nitrous oxide treated fetuses, excluding those treated on days 10 and 11, were significantly lower than those of the fed controls. The weights of the other groups did not differ significantly from the fed controls.

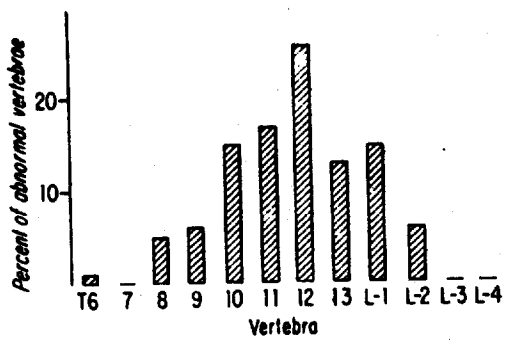

Figure 26.4. Location of abnormal vertebrae in day 20 fetuses exposed *in utero* to 70% N_2O and 30% O_2 during day 9 of pregnancy.

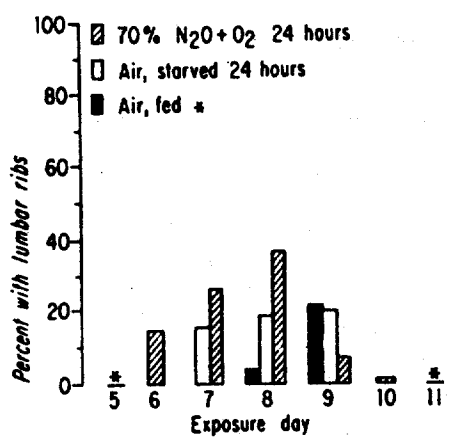

Figure 26.5. Incidence of lumbar ribs in day 20 rat fetuses. See table 26.2 for statistical analysis.

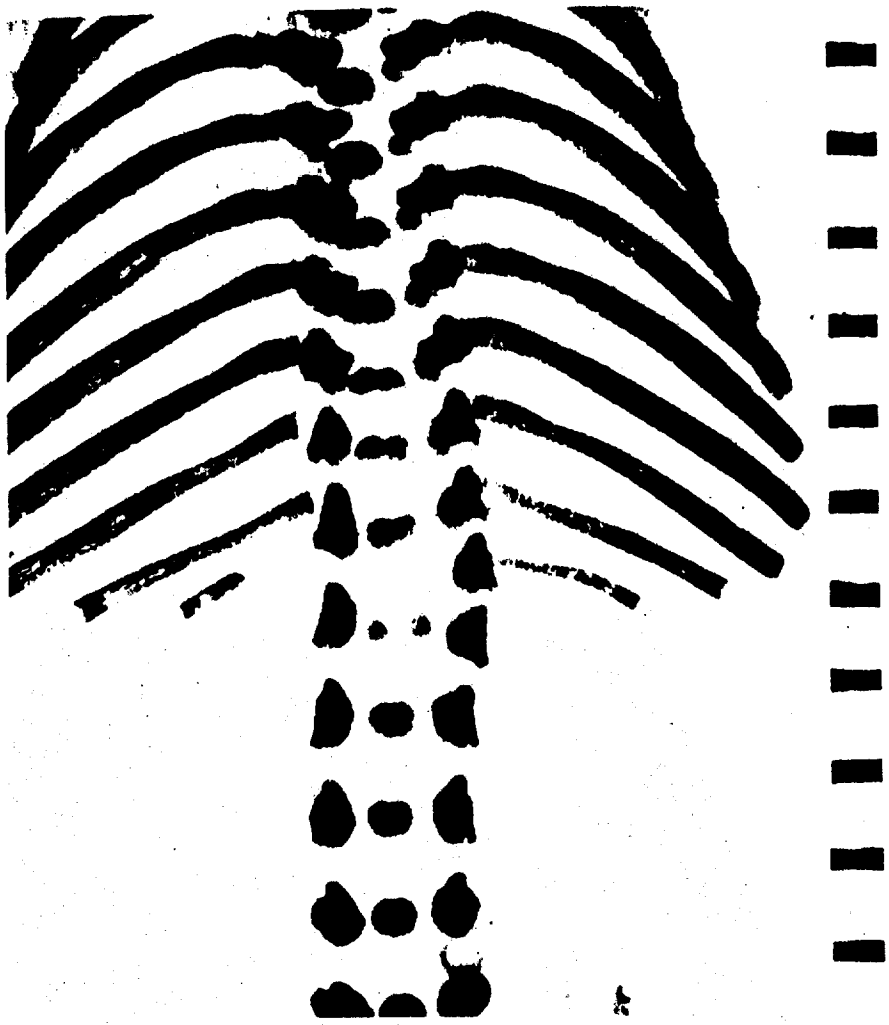

Figure 26.6. Separation of the ossification centers of the L-1 and absence of one center in T-13 is shown. There is scoleosis in the area of T-13 and part of the thirteenth rib on left is not ossified. Day 20 rat exposed to 70% N_2O and 30% O_2 on day 9. Scale in millimeters.

The control animals were placed in the same air-tight boxes and gassed with compressed air or 30% oxygen and nitrogen. The surviving fetuses from the two control gas mixtures did not differ in weight or sex ratio from the previous controls and are henceforth included in the over-all "fed" control group. Skeletons of control fetuses from rats sacrificed on days 15 through 19 were examined to determine the normal course of development of the vertebral and manubrial ossification centers. In the earliest stages of ossification, two foci could be distin-

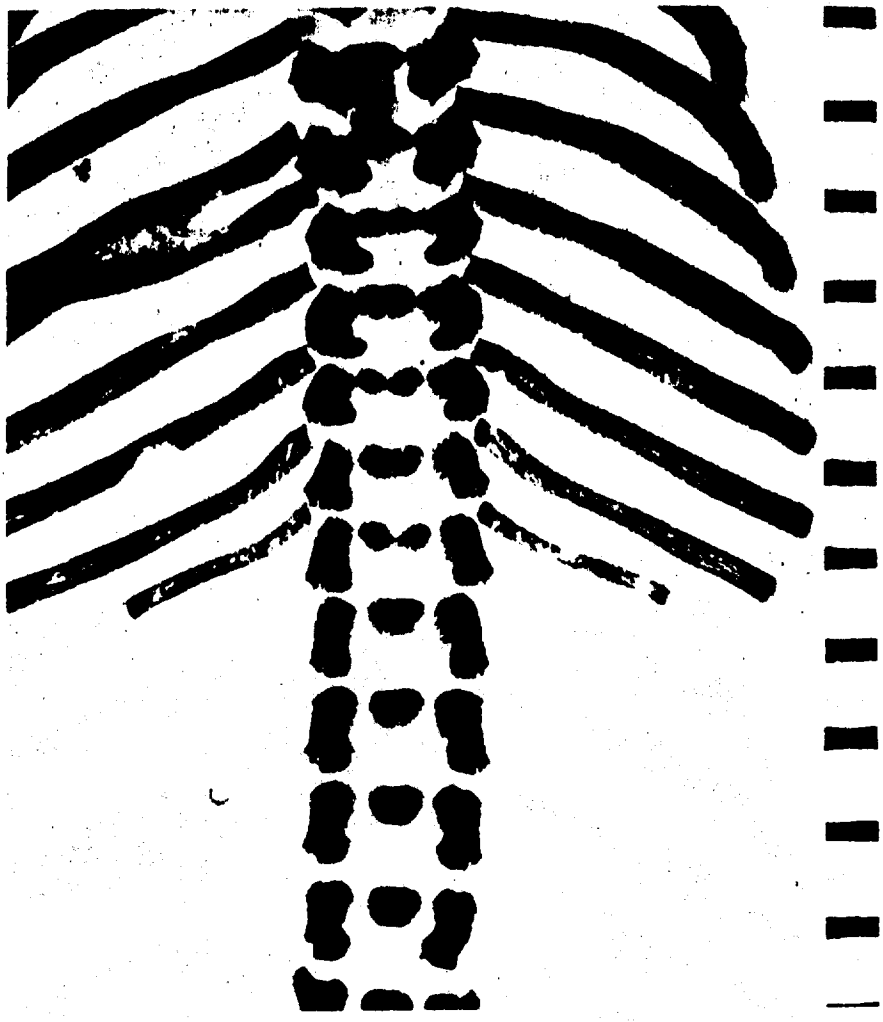

Figure 26.7. Rib fusion on day 20 rat exposed to 70% N_2O and 30% O_2 on day 9 of gestation. The dumbbell shaped vertebral centra, although more common in treated fetuses, were not classified as malformations. Scale in millimeters.

guished in the centrum of each ossifying vertebra. However, in most cases these paired centers were already partially fused at the time of their appearance, and in the majority of cases by day 19 the fusion was complete and only a single mass of ossification was visible in the centrum. In some cases dumbbell appearance attested to the original presence of two centers of ossification in a given centrum. By day 19, when the fetus averaged 2.1 g in weight, the two hemicenters were united by a dorsally located bridge of ossified tissue. Only one completely separated pair of hemicenters was found in the 19 fetuses of

day 19. Incomplete ossification of the sternum and xiphoid processes were constantly found on day 19; accordingly, little significance could be attached to the absence of one or more sternal centers on day 20. Of the fetuses in the control group, 2% had a divided center of ossification in the body of a single vertebra and 14% had supernumerary ribs attached to the first lumbar vertebra. The incidence of 14% with supernumerary ribs in the controls did not differ significantly from that in any group except those treated on day 7 or 8 with 70% nitrous oxide (table 26.2).

Because administration of 70% nitrous oxide resulted in an average

Figure 26.8. Supernumerary ribs at L-1 in a fetus exposed during day 9 to 70% N_2O and 30% O_2. Scale in millimeters.

TABLE 26.2

Weight, sex ratio and incidence of defects in rat fetuses from control, 24-hr starved, 24-hr 70% nitrous oxide exposure and 48-hr 10% oxygen exposure

Treatment	Litters	Resorptions	No. of corpora lutea of pregnancy	Survivors				Skeletal findings			
				No. surviving	No. of fetuses per litter	Average weight ± S.E.	Sex ratio*	No. of fetuses examined	Fetuses with		
									Abnormal vertebrae or ribs	Extra ribs	Less than six sternal ossification centers
						g			%		
Controls											
None	22	8	288	256	11.6	3.75 ± 0.03	129:127	161	2	14	6
Day 19	2	0	22	19	9.5	2.1		19	5	5	100
Starved controls											
Day 7	7	3	94	84	12.0	3.57 ± 0.14	38:46	71	0	15	18†
Day 8	12	7	147	128	10.7	3.59 ± 0.10	50:78	80	5	19	9
Day 9	9	5	106	94	10.4	3.66 ± 0.09	46:48	94	0	20	7
Hypoxia 10% O_2											
Days 8 and 9	3	1	38	37	12.3	3.47 ± 0.05	21:16	37	8	5	19‡
70% Nitrous oxide											
Day 5	2	1	31	29	14.5	3.07 ± 0.10§	15:14	29	3	0	59†
Day 6	5	4	83	76	15.2	3.25 ± 0.10§	35:41	76	1	14	26†
Day 7	13	2	164	161	12.4	3.05 ± 0.07§	69:92	147	14†	26†	21†
Day 8	8	2	95	91	11.4	3.36 ± 0.09§	37:54	91	25†	36†	20†
Day 9	8	8	120	108	13.5	3.37 ± 0.12§	60:48	108	54†	7	58†
Day 10	8	4	93	101	12.6	3.70 ± 0.20	55:46	78	6†	1	6
Day 11	4	4	50	42	10.5		19:23	32	3	0	
50% Nitrous oxide											
Day 8	8	7	98	77	9.6	3.52 ± 0.05	38:37	76	12†	29	5

* Ratio of males to females.

† Difference between control and treated groups significant at the 1% level or less. Calculated by the χ^2 method.

‡ Difference between control and treated groups significant at the 5% level. Calculated by the χ^2 method.

§ Difference from untreated average weight significant at the 5% level using the *t* test.

maternal weight loss of 8.0 g during 24 hr, a group of control animals was deprived of food on day 7, 8 or 9. Although the resulting day 20 fetuses were lighter, the litter size and abnormality rate of ribs and vertebrae were not increased (table 26.2; fig. 26.5). These animals lost an average of 18 g during the 24-hr starvation.

The reduction of oxygen to 10% during days 8 and 9 did not cause malformations of the skeleton (table 26.2).

An increased number of females was noted in the 7- and 8-day starved and in the 7- and 8-day nitrous oxide groups. Chi square analysis of each group, applying Yates correction for continuity (8), was significant in only the day 8 starved group ($\chi^2 = 4.0$; $p < 0.05$). When these four groups were combined and compared to the controls which were fed, the χ^2 was 8.45 ($p < 0.01$).

DISCUSSION

In the developmental stage reached by Sprague-Dawley strain embryos by the end of day 9, four somites (occipital) were present (see also Witschi (9)); during day 10, somites five through twelve (cervical) and thirteen through twenty (upper thoracic) appeared. Since the somites of vertebrae found to be affected on day 20 are not yet present at the time of exposure to nitrous oxide on day 8 or 9, we infer that the greatest effect of the nitrous oxide is concentrated in the anlage of certain still unformed somites.

The nature of the selective insult can only be guessed at, but we do not believe that maternal hypooxygenation was involved. The rats continued to feed and drink while in the nitrous oxide mixture and lost less weight than the starved controls; the color of the ears and tail remained a healthy pink. The Pa_{O_2} was measured in femoral arterial blood from two rats narcotized with the experimental 70% nitrous oxide and 30% oxygen mixture and infiltrated with lidocaine solution at the incision site over the artery. The Pa_{O_2} values were 110 and 150 mm Hg. The Pa_{O_2} in the same animals breathing air was about 100 mm Hg.

Although a deficiency in the maternal carriage of oxygen was excluded, decreased oxygen utilization at the cellular level in the embryo was not. Delay or failure in the appearance of ossification centers could result from a variety of causes, including slowed cell multiplication, cell death or disruption of enzyme induction and through a variety of molecular mechanisms. We know that the oxygen uptake of cell cultures, currently under study in our laboratories, is decreased by narcotizing concentrations of nitrous oxide. It is interesting to note that the fetal lesions we have produced with nitrous oxide are of the same type as those resulting from severe hypoxia in the hands of Ingalls (10) in mice and Dagenhardt (11) in rabbits. Axial skeletal deformities re-

sembling the nitrous oxide produced lesions have resulted from starvation of mice on day 9 (12). It may be significant that the vertebral defects are produced most readily on day 9, when the embryo probably does not possess an effective circulation for distributing oxygen and when the undifferentiated somite anlage in the region of the primitive streak, where the affected cells are presumably located, forms a focus of active cell division relatively distant from the nearest maternal oxygenated blood.

The reduced number of male offspring in rats starved or treated with nitrous oxide, on day 7 or 8, was not accompanied by a proportionate increase in fetal deaths, in contrast to the groups treated for more than a 1-day period with nitrous oxide. Until these findings are corroborated in larger groups of animals, it would be premature to ascribe the unusual sex ratio to an effect of the treatment on gonadal development.

Some of the abnormalities in ossification may legitimately be interpreted as the result of a simple delay in maturation and might not eventuate in permanent structural malformations after birth. However, the supernumerary and fused ribs and absence of hemivertebra would seem certain to result in permanent malformation; on the other hand, the end result of isolated pairs of unfused centers of vertebral ossification and absence of sternal centers cannot be predicted and requires further study which is now under way.

The relevance to human pregnancy of these observations in rats is not known. Shnider's study (13) mentions that in two out of eight instances a major anesthetic administered in the first one-third of pregnancy was followed by the birth of babies with developmental defects. Rats, of course, are not people, but they *are* mammals; the rat embryo on day 9 is at about the same stage of development as the human embryo during days 17 through 21 of gestation. Evidently more investigation is needed before the practical importance of teratogenesis by anesthetics in the human can be assessed.

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DISCUSSION AFTER PRESENTATION

Dr. Cohen: What is the shortest period of exposure of the maternal rat that will give you an appreciable incidence of vertebral defects?

Dr. Shepard: We have only reduced exposure to 24 hr so far, but we plan to keep on reducing it further until we stop seeing effects.

Dr. Cohen: I am not entirely clear as to what you feel is the interference with oxygenation in the embryo. Is this at a metabolic level or do you postulate that the delivery of oxygen is impaired?

Dr. Shepard: Obviously we don't know the mechanism, we don't know how anesthetics work. If hypooxygenation is involved I think it would be through a specific cellular mechanism rather than because of decreased carriage.

Dr. Green: The rats which are in nitrous oxide not only consume fewer calories but also become moderately dehydrated. Were your starved animals deprived of water?

Dr. Shepard: We haven't done this control yet.

Dr. Green: It is possible that dehydration might have its own effect.

Dr. Shepard: It would be a fairly mild dehydration because our rats lost only 8 g in the nitrous oxide.

Dr. Fink: May I supplement that by saying that we do, in fact, observe the amount of water that these animals drink, measuring it as inches that disappear from the water bottle, and, measured in that way, it isn't appreciably different in the rats in nitrous oxide and in the control; this makes us think that the difference in feeding of solid food is more important than the drinking water.

Dr. Finney: Regarding abnormal rib formation, since you get roughly the same effect with nitrous oxide as with starvation, do you think that it is specific to these two situations or a general response to stress?

Dr. Shepard: We do not think that differences in the incidences of supernumerary ribs in the treated and various controls are of much significance.

Dr. Smith: Concerning controls, we are using less than 24-hr exposures to the anesthetics and keep both tested and control groups away from food and water until all of the test animals are ready to eat and drink. Then both groups are allowed food and water *ad libitum*.

Dr. Brauer: The simplest feeding technique would seem to be to measure the food intake of your test population on 1 day and have your control population staggered by 1 day against this and fed comparable amounts.

Dr. Shepard: Yes, we are now including controls in which the animals are only partially starved.

Dr. Cobb: I have been impressed with the fact that most of the presentations on intact animals have included a rather long duration of exposure to noxious agents as compared to the duration of exposures that ordinarily obtain in clinical practice with humans. I should like to ask Dr. Shepard, and also Dr. Matthews and others who work with animals with very much shorter life spans than people, if it might not be appropriate to set the duration of exposure to stress as that fraction of the animal's life span which would be equivalent to the same fraction of a human life span. Might not 1 hr for the human be equivalent to 7 hr for the dog? Would it not be more appropriate to make the dog's exposure one-seventh as long as the human's and, in the case of a rat, some very much smaller fraction still?

Dr. Shepard: Most teratologists first give the animal a big dose and try to find out whether and when it is teratogenic, and then gradually reduce the duration of exposure and later the dose at the most sensitive period. Dr Basford, working with us, is down to 12 hr with some of her experiments with halothane. We intend to gradually reduce the exposure times to nitrous oxide. Now I don't think I would accept your suggestion of going by the life span of the animal. I think if you are going to make such analogies it is better to consider the duration of the embryonic period, how long, for instance, the rat takes to go through a particular stage as compared to the human. Dr. Matthews, do you want to comment on that?

Dr. Matthews: I just want to say that the cell cycle is approximately the same length in all animals. It is really the cell cycle, the mitosis rate, that we are modifying in these animals too.

Dr. Shepard: We have no evidence at all that the malformations are related to disturbances of mitosis. We might have cell death, or all sorts of other things.

Dr. Wilson: It seems to me that metabolic factors might be more significant than any of these arbitraty time considerations. We have time for one more question.

Dr. Bruce: I was interested in Dr. Green's observation yesterday that there is a strain difference among rats with regard to the effects of the hemopoietic system. Have you made any observations of possible strain differences in your tests?

Dr. Shepard: Rather than look for strain differences in rats I would like to see what happens in the primate; we have not yet adequately assessed the effect of anesthetics on human pregnancy. If we had enough pregnant patients receiving anesthesia we would do a prospective study and X-ray the children that were born to see if they had supernumerary ribs or abnormalities of the spine.

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In each issue of the Journal an article of particular interest and value to anesthesiologists, which has been published elsewhere, will be presented. Primarily the material will contain fundamental basic information which has been presented by anesthesiologists, physiologists, pharmacologists, and others and, on occasion, by commercial firms. In the last instance, these reprintings are not to be construed as an endorsement of the particular commercial firm or its product by Anesthesia and Analgesia—Current Researches.

Nitrous Oxide IN THE FOOD AND AEROSOL INDUSTRIES

C. A. SIMPLER*
Madison, Wisconsin

JOSEPH PRIESTLEY, the English clergyman-chemist, first identified nitrous oxide in 1772. Were he alive today, he would scarcely recognize this gas as an ingredient of those fluffy blobs of topping served on ice cream sundaes, sodas, and malted milk drinks, and in many other types of aerosol-propelled food products. Nor would the anesthesiologists of a century ago, who first used "laughing gas," have anticipated its later importance in the food industry.

Nitrous oxide, or dinitrogen monoxide (N_2O), is manufactured commercially by the thermal decomposition of ammonium nitrate. Byproducts of the decomposition are water, nitrogen, and higher oxides of nitrogen such as NO (nitric oxide), and NO_2 (nitrogen dioxide). Most of the higher oxides of nitrogen, unlike nitrous oxide, can be hydrated to acids and react with sodium hydroxide to form soluble salts which can be removed from the gas stream.

*Vice President-Sales, Ohio Chemical & Surgical Equipment Co., Madison, Wisconsin.

Water is removed by chemical dryers, and free nitrogen is removed by fractional distillation of liquefied nitrous oxide.

Nitrous oxide has an oxidizing potential between pure oxygen and air, and will support combustion. However, it is an extremely stable compound at room temperatures and dissociates only at temperatures exceeding 1200° F. At temperatures under 392° F., metals such as iron, copper, and zinc are unaffected by nitrous oxide.

The boiling point of nitrous oxide is 127.5° F., and its latent heat of vaporization is rather high, about 161.8 B.T.U. per pound. It is soluble in water, and is normally handled as a liquid in high-pressure cylinders. Pressure in these cylinders at 70° F. is 745 pounds per square inch gauge. It is also available for cryogenic use and can be transported and stored at or near its boiling point.

Certain of the properties of nitrous oxide—such as high solubility, chemical stability, its faintly sweet taste, and its acceptability in foods—lend themselves ideally to its use as a propellant in aerosol packaging. For many years nitrous oxide has been used as the propellant and whipping agent for pressurized whipped cream and, more recently, for vegetable-based whipped toppings. In such products, the can is filled and the cap and valve crimped in place. Then the valve is opened and the can charged with N_2O, under vigorous agitation to facilitate absorption of the gas. In use, the gas pressure forces the product-gas mixture out of the valve. On emergence into the reduced pressure of the atmosphere, from a container pressure of perhaps 100 pounds per square inch gauge pressure, the N_2O is released from solution, resulting in the foaming, or "whipping," of the product.

Use in Other Preparations — Much work has been in progress in recent years on the development of other food aerosols. Such proposed products include canape spreads, salad dressings and mayonnaise, and new whipped toppings and syrups. In some cases, such as in barbecue-sauce aerosol, a spray pattern is called for instead of an aerated stream. Nitrous oxide used as a propellant can also produce this spray effect with different product characteristics using different valving arrangements.

The same properties that make nitrous oxide so suitable for use in aerosol packaging make this gas an ideal medium for the aeration of fluffing of such food products as fluffy marshmallow, ice cream, and peanut butter.

A significant portion of the food packaging field has found the use of flexible packaging as a lightweight, easy-to-ship, easy-to-use means of packaging food items. The applications in the field are varied and many, and include items such as dehydrated potatoes, sliced cheese, and "boil-in-the-bag" items. Many of these items are packaged in an atmosphere of inert gas as a means of excluding oxygen, which would cause the rapid deterioration of the product and loss in color, flavor, odor, and vitamin content.

Employment as "Tracer Gas"—Leakage in a flexible package is extremely difficult to detect by conventional methods. Presently under development is a method utilizing N_2O as a tracer gas. Nitrous oxide is present only as a trace (0.00005 per cent by volume) in the atmosphere. It is therefore unique among the gases commonly used for leak detection and can be readily or easily traced. Basically, the system involves the mixing of a small amount of N_2O as part of the packaging gas; any leakage of the nitrous oxide from the flexible package would be detected by an infrared apparatus, which is capable of finding a few parts of N_2O in a million parts of background gas. This system, which is adaptable to a continuous processing and testing line, is nondestructive to the package and is therefore economical to use.

Recently this gas has found an interesting application as a tracer gas in the

location of leaks in natural gas transmission lines. Very low concentrations of the gas are dissolved in the water used for hydrostatic pressure testing of the pipes. In the event of a leak, the nitrous oxide-laden water escapes from the pipe, the liberated nitrous oxide making its way to the surface of the ground where its presence in trace quantities can be determined by infrared sensing equipment.

Use in Frozen Food Production — In the production of frozen foods, it is recognized that the more rapid the freezing rate, the better the quality of the product. With faster freezing, crystal size is smaller. This and other related factors minimize the cell damage and so-called extracellular freezing which, in the conventional slow-freezing processes, result in mushiness and drainage, with accompanying losses in flavor and appearance.

The most effective means of withdrawing heat from a product is by immersion of the product directly into a liquid refrigerant or a refrigerated medium. The physical characteristics of N_2O lend themselves well to this freezing technique. Its boiling point is sufficiently low for rapid freezing, and its relatively high heat capacity contributes to the efficiency of the process.

Under development is a freezing process involving the immersion of a food product directly into a bath of liquid N_2O at a temperature of −127.5° F. With this process, freezing rate is reduced from a period of hours, required with conventional practices, to a matter of minutes. Improvement in quality of such products as frozen strawberries and cherries is dramatic. Obvious improvement in quality can also be noted in such products as shrimp and other seafood, meats, and even frozen baked goods. A number of new products have been made possible by this new type of ultra-low-temperature freezing.

There are certain beverages which could benefit from effervescence, but in which acid formation would be detrimental from a flavor standpoint, or perhaps from the effect of the acid on the ingredients or on the container. Nitrous oxide is being investigated as a means of "charging" these beverages. While it can theoretically be considered the anhydride of hyponitrous acid, this point is purely academic, since hyponitrous acid cannot exist in water and simply dissociates to water and N_2O. Therefore, a solution of N_2O in water brings about no change in pH.

Chemists and technicians are constantly studying new uses of nitrous oxide. In commercial use, "laughing gas" is becoming a "wonder gas" in the number of its contributions to better living in this modern age.

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THE RELATIVE ANAESTHETIC ACTIVITY OF THE BUTANES AND PENTANES

ROGER W. STOUGHTON AND PAUL D. LAMSON

From the Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee

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Of the straight-chain, saturated hydrocarbons methane (1), propane (2), pentane (3, 4), hexane (3, 4), heptane (3, 4), and octane (3, 4) have been examined for their anaesthetic properties. Butane (5), although investigated as an industrial hazard, has not been studied as an anaesthetic.

With the exception of 2-methyl hexane (4), we have been unable to find reference to anaesthetic studies on branched saturated hydrocarbons.

The work here reported deals with all the isomers of butane and pentane—butane and isobutane, and pentane, isopentane and neopentane. Ether and cyclopropane were used for the sake of comparison. The experiments here reported were carried out on mice. A few dogs were also anaesthetized with some of these substances. The time taken to produce "light anaesthesia" was determined by placing two mice in a 2-liter bottle filled with a gas mixture of known concentration, and revolving the bottle mechanically at fourteen revolutions per minute. The endpoint used was the time taken to produce that degree of anaesthesia in which the mouse was unable to maintain an upright position. "Complete anaesthesia" was obtained by placing five mice at one time in a 20-liter bottle containing the gas mixture, in which was suspended a bag of soda lime, and determining the time taken to cause inability to regain an upright position after shaking the bottle. Lethality was determined by noting the percentage of mice that died after two hours exposure in this bottle to a given gas concentration. Our results obtained on pentane by these

TABLE 1

Anaesthetic action of various substances

Compound	Concentration: Millimoles per liter	Concentration: Volume	Average onset of: Light anaesthesia	Average onset of: Loss of posture	Lethality: Per cent dead in 2 hours	Lethality: Average time of death	Average time of recovery	Number of mice
		per cent	*minutes*	*minutes*		*minutes*	*minutes*	
n-Butane	5.8	13	25					6
	9.8	22	1	15	0		1	10
	12.0	27		4	40	84	5	10
	13.8	31		3	60	65	3	10
Isobutane	6.7	15	60					6
	8.9	20	17					6
	11.3	23	26					6
	15.6	35		25	0		3	10
	17.9	41		3	60	72	4	10
	23.1	53		2	100	28		10
n-Pentane	3.0	7	10					6
	3.5	8	3					6
	4.2	9	1.3	8.2	0		4	10
	4.5	10		4.5	45	73	8	11
	4.9	11		2.0	90	62	7	10
Isopentane	4.2	9	11.6					6
	4.9	11	3.9	10.0	0		4	6
	5.4	12	2.2					6
	5.8	13		5.0	20	94	8	10
	6.3	14		3.4	50	84		10
Neopentane	8.9	20	30	∞	0		0.1	5
	12.0	27		3.0	0		2	5
	15.2	34		2.0	40	35	4	5
Ether	1.5	3.5		15.0	0		5	12
	2.0	5	3	6.5	90	64		12
	2.5	6		3.0	100	59		12
Cyclopropane	5.8	13		3.0	0		1.5	10
	6.7	15		1.5	40	81	3.5	10
	8.0	18		1.25	90	39		10

methods check very well with the previous work of Fühner and Lazarew.

In the case of neopentane, which was kindly given to us by Dr. Frank C. Whitmore of Pennsylvania State College, the amount available (9 grams) did not allow as many experiments, but by using a 5-liter bottle we were able to make three determinations on five mice each. The results of these studies are given in table 1.

All of these substances produce considerable excitement in mice during anaesthesia—cyclopropane and isopentane the least, ether and normal butane more, isobutane and normal pentane still more. Neopentane differed from the others in causing still more excitement and a general cramp-like effect just before anaesthesia was induced.

On removal from the jar after two hours anaesthesia the mice recovered very quickly, usually in one or two minutes, running about normally except after ether where recovery was definitely delayed. Those recovering from neopentane showed marked hyperexcitability when stimulated by noise or touch. All mice that did not die during the period of anaesthesia remained normal for the twenty-four to forty-eight hours observed after anaesthesia.

Pentane was definitely more active as well as more toxic than butane, which fits in with the findings of Fühner and Lazarew who found an increasing activity and lethality as the number of carbon atoms was increased.

The effect of branching in these compounds was quite striking. Isobutane was less active and less lethal than normal butane. Isopentane was also less active and less lethal than normal pentane, while neopentane was much less active and caused fewer deaths in the same concentration than either pentane or isopentane. There was, however, no correlation between the degree of branching and the degree of excitability.

A few experiments on dogs were carried out with butane, isobutane, and isopentane. Although enough experiments were not done to give accurate, quantitative results, we found that it was extremely difficult to produce good anaesthesia and relaxation with any of them, while control experiments with cyclopropane were carried out with great ease.

Anaesthesia was induced by using a gas-oxygen machine, mask, soda-lime tube, and gas bag. A Magill catheter with Guedel inflatable cuff was then introduced and the dog connected with a spirometer containing the gas mixture. In order to avoid the high initial concentration necessary for insertion of the cannula, some dogs were anaesthetized by forcing their heads through a rubber membrane into a specially devised gas chamber in which the anaesthetic concentration could be controlled at will.

None of these gases gave good anaesthesia and relaxation except in concentrations which were practically lethal. Butane required a concentration of about 20 to 25 per cent for relaxation, but this caused death after a short time. Isobutane required about 45 per cent for anaesthesia, and death occurred with 55 per cent. Isopentane caused fair anaesthesia with about 12 per cent and death with 15 to 17 per cent. A much more extensive pharmacological study together with blood analyses would be necessary to determine definitely the practical value of these gases as inhalation anaesthetics, but our experience would lead us to believe that there is only a small margin of safety between the anaesthetic concentration and that causing death.

CONCLUSIONS

1. The anaesthetic activity and lethality of butane fall below that of pentane.

2. Branched isomers of the saturated hydrocarbons, butane and pentane, showed less activity as well as lethality than the straight-chain compounds, isobutane being less active and less lethal than butane, and the series, pentane, isopentane, and neopentane, showing this effect very strikingly.

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