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EFFECTS OF CARBON MONOXIDE ON BRAIN CELLULAR METABOLISM IN MONKEYS

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The toxic effects of carbon monoxide in terms of atmospheric contamination and industrial air pollution control have been extensively studied. Since CO is one of the major toxic products found in various operational situations as well as in aerospace systems, the Air Force also has a prime interest in determining the effects of continuous relatively low level exposures to CO.

Groups of monkeys were exposed to atmospheres of 0, 220 mg/M^3 and 440 mg/M^3 CO in 68% oxygen 32% nitrogen at 5 psia and the effects of these atmospheres on brain mitochondrial respiration and energy production and on blood gas levels were studied. There were no significant changes in mitochondrial respiration or energy production. There was a significant decrease in blood oxygen content, oxygen saturation and pO_2 in monkeys exposed to 440 mg/M^3 CO.

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FOREWORD

This study was performed in support of Project 7163, "Research on Biomechanisms and Metabolism." The work was performed from November 1968 to June 1969 in the Toxicology Branch, Toxic Hazards Division. One phase of this work was performed by personnel of the Illinois Institute of Technology Research Institute, Chicago Illinois, under Air Force Contract F33615-68-C-1270.

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INTRODUCTION

The toxicity of carbon monoxide has been extensively studied in many animal species and in man. The current interest is primarily concerned with air pollution and with establishing standards and controls for community and industrial atmospheric contamination. The effects of low level exposure to various air pollutants, including carbon monoxide, on the general population, i.e., people of all age groups in varying states of health and disease, are receiving wide attention.

The Air Force also has a primary interest in possible toxic effects of carbon monoxide, but on a relatively young healthy male population functioning under unique and specific conditions. It has been shown that CO is one of the major toxic products found in many operational situations as well as in high performance aircraft and aerospace systems in which men must continuously function at a high level of performance. There is, therefore, a potential risk in exposure to continuous low level concentrations of CO which cause no subjective or objective symptoms but which may affect man's ability to think or function normally.

The toxic action of CO is caused principally by the combining of CO with hemoglobin resulting in hypoxia. CO has approximately 200 times more affinity for hemoglobin than does oxygen, the

oxyhemoglobin is replaced with carboxyhemoglobin (COHb), and oxygen transport to the tissue is decreased. Furthermore, there is a shift of the oxygen dissociation curve to the left causing an impaired release of oxygen from oxyhemoglobin for diffusion into the tissues. It has been reported by Lapresle & Fordeau (1967) that the central nervous system is the most sensitive to CO intoxication. The effects on the brain are similar to those found with severe hypoxia. Pathologic examination of the brains of humans who died of acute CO intoxication showed edema, hemorrhage, and areas of necrosis principally in the globus pallidus, Ammon's horn of the hippocampus, and the subcortical white matter. The exact concentration and duration of exposure to CO required to produce toxic effects is questionable since the literature contains many conflicting reports. Trouten and Eysenck (1961) reported difficulty in limb coordination in dogs with COHb levels of 5-10%. Lewey & Drabkin (1944) also reported disturbances in gait and reflexes of dogs exposed to 115 mg/M^3 CO for 6 hours a day, 6 days a week for 11 weeks. On autopsy they found necrosis and demyelinization in the white matter of the cerebral hemispheres, globus pallidus, and brain stem. Lindenberg et al (1962) exposed groups of dogs both intermittently and continuously for 6 weeks at 58 mg/M^3 CO and found glial mobilization and dilation of the lateral cerebral hemispheres.

However, dogs, monkeys, and rats exposed to 220 mg/M^3 and to 440 mg/M^3 CO in 68% oxygen, 32% nitrogen at 5 psia for 168 days did not exhibit similar pathologic changes (Theodore et al 1970). Musselman et al (1959) reported no toxic effects in dogs, rats or rabbits continuously exposed to 50 ppm CO for 3 months. There also have been many performance studies reported with somewhat conflicting results. Beard and Wertheim (1967) reported performance decrement in humans with COHb of 3-5%. Schulte (1963) applied a battery of tests to human subjects breathing concentrations of CO sufficient to raise their COHb as high as 20% and found significant psychomotor impairment in subjects with COHb levels as low as 3%. MacFarland et al (1944) showed an impairment in discrimination of subjects with 14% COHb. Conversely, monkeys exposed to 220 mg/M^3 CO and to 440 mg/M^3 CO in an atmosphere of 68% oxygen, 32% nitrogen at 5 psi and with COHb levels of 20% and 30% respectively showed no performance decrement in continuous and discrete avoidance tasks (Back, 1969; Theodore et al, 1970). Mikulka et al (1969) exposed human subjects to 50 ppm, 125 ppm, 200 ppm or 250 ppm for three hours, measured a broad range of performance tasks and found no decrement.

Very little work has been done on the effects of CO at the cellular level. Estler (1961) measured glycolysis in brain tissue of mice exposed to high levels of CO for short time periods. He

found glycolysis was stimulated, and creatine phosphate decreased indicating insufficient production of energy. It has been demonstrated that marked hypoxia depresses cerebral metabolism but mild hypoxia causes little metabolic change (McIlwain 1966). When the oxygen content of the blood is reduced, there is a compensatory increase in blood flow. However, since reduced oxygen availability affects various parts of the brain differently, there may be localized metabolic changes of the areas most sensitive to oxygen deprivation (Van Liete & Stickney 1963). In general with a decrease in oxygen to 10% there is an increased glycolysis, inorganic phosphate increases and creatine phosphate decreases.

The question arises then whether the degree of hypoxia produced by low level continuous exposure to CO would produce metabolic changes in the brain.

The study reported here was undertaken to determine if continuous CO exposure would cause changes in brain tissue at the cellular and subcellular level in the absence of any overt clinical signs and to establish and measure any impairment in cellular respiration and high energy production. Relatively high concentrations of CO were used initially in an attempt to induce changes with succeeding exposures to be at lower concentrations. The two exposure concentrations of CO described in this paper were 220 mg/M^3 and 440 mg/M^3 in the atmosphere used for spacecraft;

namely, 68% oxygen, 32% nitrogen at 5 psia. Since we have found an increase in total hemoglobin, hematocrit and erythrocyte count in dogs and monkeys after exposure to 550 mg/M^3 CO, two exposure periods, 5 and 14 days, were chosen to establish if an erythropoietic response was occurring over a period of time and possibly modifying any toxic effects.

METHODS

Eighteen monkeys, Macaca mulatta, ranging in weight from 2.3 to 4.5 kilograms (k), were divided into three groups of six monkeys each. Group I was exposed to an atmosphere of 68% oxygen, 32% nitrogen at 5 psia; Group II was exposed to 220 mg/M^3 CO in the same atmosphere; and Group III was exposed to 440 mg/M^3 CO in the same atmosphere. The three groups were further subdivided so that three monkeys in each group, IA, IIA, IIIA, were exposed to the respective experimental atmospheres for 5 days and the other three monkeys in each group, groups IB, IIB, IIIB, were exposed 14 days (Table 1).

All exposures were conducted in the Thomas Domes (Thomas 1965) which are dynamic flow experimental altitude chambers. The exact dome environmental conditions are listed in Table II. At a flow rate of 40 cu ft/min, there was a complete change of atmosphere about every 20 minutes. The monkeys were housed in individual cages equipped with an automatic watering system and were fed once a day with Purina(R) monkey chow. The domes are equipped with airlocks which permit entry for feeding and cleaning purposes and for accomplishing the experimental procedures without changes in the experimental atmosphere.

Prior to being placed in the dome, all monkeys underwent

surgery to remove two circular areas of bone from the skull in order to facilitate obtaining brain tissue after exposure. Two bilateral bone plugs, approximately 2.0 centimeters (cm) in diameter were removed from the parietal bones 0.5 cm lateral to the sagittal suture and 0.5 cm anterior to the lambdoid suture. This was done without damage to the dura. An inert plastic sponge material[1] was carefully fitted into the cavity without pressure on the brain and the skin flaps sutured over the wound. The monkeys were allowed to recover four weeks before being placed in the dome and the experimental atmosphere. At the end of the exposure periods and while still in the exposure atmosphere, blood was drawn under local anesthesia from the femoral artery for determination of oxygen content, capacity, and saturation, pO_2, CO_2 content, pCO_2, pH, total hemoglobin, hematocrit, and carboxyhemoglobin. The monkeys were then anesthetized with intravenous sodium pentobarbital, 30 mg/k. The skin over the skull openings was laid back and the foam plugs removed exposing the cerebral hemispheres.

Tissue from the right cerebral hemisphere was taken for determination of creatine phosphate, CP; adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenosine monophosphate, AMP; inorganic phosphate, Pi; and pyridine nucleotides, reduced and

1. The exact chemical composition of this material is not available since it is no longer in production.

oxidized, NAD, NADH, NADP, NADPH. Since these compounds are extremely labile, the tissue was frozen almost instantly in situ using the method described by Hohorst (1959). Sharp pointed tongs precooled in liquid nitrogen were quickly thrust into the brain and approximately 400 mg of tissue clamped, excised, and immediately placed in liquid nitrogen. In a cold room maintained at -20°C approximately 75 mg of the frozen tissue were wrapped in aluminum foil and stored in dry ice pending analysis of pyridine nucleotides. The remaining tissue was powdered under liquid nitrogen, weighed and extracts prepared essentially as described by Lowry et al (1963). These extracts were stored in dry ice until analyzed. Protein content of the tissues was determined by the alkaline copper tartrate reaction (Lowry 1951) standardized against serum bovine albumen. The adenylic and pyridine nucleotides were measured by fluorometric techniques (Lowry et al 1963).

Immediately after the frozen tissue was obtained and placed in liquid nitrogen, tissue from the left hemisphere was obtained for determination of respiratory control indices and ADP/O ratios on mitochondrial preparations. Approximately 4.5 g of tissue were scooped out and placed immediately in an ice cold solution of mannitol, 0.3M, pH 7.4, containing 0.1 mM EDTA, and minced. The mitochondria were prepared by the method of Ozawa et al (1966) with slight modifications.

The tissue was weighed in 30 ml mannitol solution and homogenized at 0°C in an ace tissue grinder for 30-45 seconds. The homogenate was centrifuged at 0°C in a refrigerated centrifuge at 600x g for 8 minutes. The supernatant was decanted and spun at 10,000x g for 10 minutes. The supernatant was discarded and the pellet washed with mannitol solution. The pellet was resuspended in mannitol solution and centrifuged at 5,000x g for 10 minutes. The supernatant was discarded, precipitate washed, and the washed pellet suspended in a volume of mannitol solution equal to 0.2 times the original tissue wet weight. The mitochondrial suspension (0.1 ml) was incubated at pH 7.4 in 1.5 ml media containing; 300 uM mannitol, 5 uM orthophosphate, 10 uM KCl, 10 uM Tris, 0.2 uM EDTA, 10 uM substrate per ml. There were three substrates used: glutamate, succinate, and α-ketoglutarate. The temperature was maintained at 25°C $\pm$ 0.5°C using a constant temperature circulating water bath. The oxygen uptake was measured using a Gilson oxygraph with a Clark oxygen electrode.[2] The oxygen uptake was measured for a short time in this condition, State 4; and then ADP, 0.16 uM/ml was added and the oxygen uptake measured in the presence of phosphate acceptor, State 3. This cycle was repeated two times and the results of

2. Yellow Springs Instrument Company

cycle 2 and 3 averaged for calculation of RCI and ADP/O. Protein analyses of mitochondria were also performed (Lowry, 1951).

After the tissue had been obtained for analysis the monkeys were killed and necropsies performed.

Table 1
Exposure Conditions

Group	Number of Monkeys	Days Exposure	Exposure Conditions
IA	3	5	68% oxygen, 32% nitrogen 5 psia
IB	3	14	68% oxygen, 32% nitrogen 5 psia
IIA	3	5	68% oxygen, 32% nitrogen 5 psia 220 mg/M^3 CO
IIB	3	14	68% oxygen, 32% nitrogen 5 psia 220 mg/M^3 CO
IIIA	3	5	68% oxygen, 32% nitrogen 5 psia 440 mg/M^3 CO
IIIB	3	14	68% oxygen, 32% nitrogen 5 psia 440 mg/M^3 CO

Table II

Dome Operating Conditions

	Control		220 mg/M^3 CO		440 mg/M^3 CO	
	Mean	Range	Mean	Range	Mean	Range
Pressure mmHg	260	--	260	--	260	--
O_2 Partial Pressure mmHg	177	--	177	--	177	--
Flow rate cu ft/min	40	--	40	--	40	--
Temperature °F	74	65-78	76	70-84	73	58-77
Relative Humidity	39	19-86	49	22-100	28	18-100
CO_2 %	0.02	0.00-0.03	0.02	0.00-0.15	0.03	0.00-0.24
CO mg/M^3	0	0	222	215-232	440	345-484

RESULTS

All monkeys appeared healthy throughout the exposures and showed no clinical signs of toxicity.

The blood gas data are shown in Table III. Blood samples were obtained from 5 monkeys in Groups I and II and 4 monkeys in Group III. There were no significant differences between monkeys exposed for 5 days and those exposed for 14 days within any group; therefore, the data for Groups A & B were combined. The blood gases and hematologic response had apparently stabilized by 5 days and showed no further change in 14 days. There were no significant differences between Group I and Group II except for the level of carboxyhemoglobin, 20.2% $\pm$ 1.1%. However, the animals in Group III exhibited significant decreases in oxygen content, oxygen saturation, and pO_2 compared to monkeys in both Groups I and II ($P<.01$). Unfortunately, the carboxyhemoglobin analyses of the blood samples from monkeys exposed to 440 mg/M^3 CO were unsatisfactory. Twelve monkeys of approximately the same age and weight exposed to the same atmosphere in an adjoining dome showed a mean carboxyhemoglobin level of 30.2% $\pm$ 1.5%. Several experiments in this laboratory have indicated that carboxyhemoglobin levels in monkeys exposed to the same concentration of CO plateau after an equilibration period of 24-48 hours and do

not vary appreciably thereafter.

The results of the CP, ATP, ADP, AMP and Pi determinations are listed in Table IV. There were no significant differences between monkeys exposed for 5 or 14 days within any single group. No significant differences were found between Groups I and II. There did appear to be a trend in Group III toward an increase in ADP, AMP, and Pi with a concommitant decrease in C.P. However, no significant differences were found between Group III and Groups I and II.

The mean pyridine nucleotide levels are listed in Table V. There were no significant changes between any groups.

Tables VI lists the mean Q(O/P) in State 3 and State 4. There were no significant differences between any of the groups in State 3 or in State 4. Results of the ADP/O and RCI are shown in Table VII. There were no significant differences between Groups II and III and Group I.

The results of the necropsies showed there were no pathological changes due to exposure to CO in any of the organs examined. Several monkeys had mild lung mite infestation. There was no chronic inflammatory reaction associated with the surgical procedures used to remove the bone plugs from the skull prior to exposure.

Table III

Blood Gas Analysis

	Group I	Group II	Group III
O_2 content vol %	13.86 ± 1.05	13.34 ± 0.79	10.01 ± 1.02[b]
O_2 capacity vol %	16.18 ± 1.17	17.61 ± 0.85	17.93 ± 1.73
Saturation %	85.8 ± 6.1	75.7 ± 2.7	57.2 ± 2.7[b]
pO_2 mmHg	81.9 ± 8.4	75.1 ± 5.5	64.6 ± 4.6[b]
CO_2 content vol %	27.3 ± 5.4	26.7 ± 2.8	25.7 ± 6.5
pCO_2 mmHg	24.7 ± 7.2	24.8 ± 2.8	21.2 ± 3.7
pH	7.374 ± 0.049	7.374 ± 0.075	7.322 ± 0.057
COHb %	<0.5	20.2 ± 1.1	30.2 ± 1.5[a]
Hemoglobin g %	12.1 ± 0.6	13.1 ± 0.7	
Hematocrit %	42.5 ± 2.1	45.0 ± 2.5	

a. Historical controls - The samples were unsatisfactory. This is the mean % carboxyhemoglobin of 12 monkeys under the same exposure conditions.

b. Group III monkeys significantly lower than Group I and Group II, $P<.01$

Table IV

Results of Biochemical Analyses on Frozen Brain Tissue

	C.P.	ATP	ADP	AMP	Pi
Group I	47.5 ± 12.9	20.9 ± 3.9	7.01 ± 2.14	1.65 ± 1.54	37.8 ± 10.0
Group IIA	58.4 ± 9.5	24.8 ± 3.2	6.49 ± 0.72	1.23 ± 0.64	33.6 ± 8.7
Group IIB	55.1 ± 9.1	23.3 ± 2.6	5.54 ± 1.28	1.33 ± 0.36	33.7 ± 8.9
Group II	56.8 ± 8.5	24.0 ± 2.8	6.02 ± 1.07	1.28 ± 0.47	33.6 ± 7.9
Group IIIA	36.0 ± 26.2	20.5 ± 9.0	10.29 ± 2.02	2.61 ± 1.37	54.6 ± 3.5
Group IIIB	38.0 ± 23.0	21.5 ± 2.5	6.52 ± 1.87	1.45 ± 0.97	46.5 ± 23.5
Group III	37.0 ± 22.1	21.0 ± 5.9	8.41 ± 2.70	2.03 ± 1.24	50.6 ± 15.7

Table V

Pyridine Nucelotides

muM/g

	NAD	NADH	NADP	NADPH
Group I	359 ± 80	21 ± 8	22.8 ± 7.7	26.9 ± 4.2
Group IIA	226 ± 72	24 ± 4	22.3 ± 0.8	30.9 ± 3.1
Group IIB	391 ± 27	40 ± 22	22.3 ± 0.4	27.9 ± 3.6
Group II	309 ± 108	32 ± 17	22.3 ± 0.75	29.4 ± 4.0
Group IIA	350 ± 39	46 ± 17	22.7 ± 3.8	26.7 ± 5.2
Group IIB	324 ± 67	33 ± 2	20.5 ± 0.8	24.0 ± 4.9
Group III	337 ± 62	41 ± 16	21.6 ± 3.2	25.4 ± 5.8

Table VI
Oxygen Uptake of Mitochondria in State 3 and State 4

	Q(O/P) State 3 mu atoms O/mg protein/min			Q(O/P) State 4 mu atoms O/mg Protein/min		
	Glutamate	Succinate	α-Ketoglutamate	Glutamate	Succinate	α-Ketoglutarate
Group I	59.8 ± 13.9	84.5 ± 12.4	30.4 ± 7.3	13.7 ± 2.5	33.7 ± 8.1	15.7 ± 2.5
Group IIA	60.7 ± 8.6	90.4 ± 11.1	29.1 ± 0.8	17.5 ± 4.0	41.6 ± 6.5	20.2 ± 4.5
Group IIB	68.9 ± 11.8	99.0 ± 4.4	39.1 ± 8.4	17.9 ± 3.0	44.1 ± 8.6	21.8 ± 6.4
Group II	64.8 ± 10.2	94.7 ± 8.9	34.1 ± 8.5	17.7 ± 3.2	42.8 ± 7.0	21.0 ± 5.0
Group IIIA	71.3 ± 8.1	103.6 ± 18.7	37.0 ± 6.4	19.2 ± 3.2	44.4 ± 6.5	20.7 ± 2.9
Group IIIB	69.1 ± 15.1	97.4 ± 19.3	39.1 ± 10.1	15.3 ± 3.1	34.2 ± 6.4	17.0 ± 3.2
Group III	70.2 ± 10.9	100.5 ± 17.3	38.1 ± 7.7	17.3 ± 3.5	39.3 ± 8.0	18.9 ± 3.4

Table VII
Mitochondrial Respiration

	ADP/O			RC		
	Glutamate	Succinate	α-Ketoglutarate	Glutamate	Succinate	α-Ketoglutarate
Group I	2.13 ± 0.13	1.52 ± 0.10	1.77 ± 0.33	4.44 ± 1.18	2.58 ± 0.50	2.14 ± 0.71
Group IIA	2.09 ± 0.07	1.55 ± 0.11	1.50 ± 0.37	3.53 ± 0.36	2.53 ± 0.40	1.49 ± 0.30
Group IIB	2.13 ± 0.09	1.53 ± 0.08	1.67 ± 0.07	3.91 ± 0.41	2.31 ± 0.46	1.81 ± 0.09
Group II	2.11 ± 0.08	1.54 ± 0.06	1.59 ± 0.25	3.72 ± 0.40	2.42 ± 0.40	1.65 ± 0.27
Group IIIA	2.14 ± 0.08	1.50 ± 0.04	1.67 ± 0.10	3.95 ± 0.56	2.33 ± 0.16	1.78 ± 0.06
Group IIIB	2.23 ± 0.03	1.54 ± 0.06	1.90 ± 0.11	4.52 ± 0.31	2.84 ± 0.10	2.30 ± 0.46
Group III	2.19 ± 0.07	1.52 ± 0.05	1.78 ± 0.16	4.24 ± 0.51	2.58 ± 0.31	2.04 ± 0.41

DISCUSSION

It must be emphasized that these data are preliminary and are based on a very limited number of animals. The initial intent was to establish the minimum concentration of CO at which an effect could be seen within a short period of time and then to expose a larger group of monkeys to that concentration. This report is an evaluation of the first two concentrations used and of two exposure time periods.

We were basically interested in determining if changes in subcellular metabolism would occur in response to continuous exposure to 220 mg/M^3 or 440 mg/M^3 CO; that is, whether the amount of carboxyhemoglobin formed at these exposure levels, 20% and 30% COHb respectively, would produce a degree of hypoxia necessary to affect mitochondrial respiration and energy production. Also, we wished to establish if these changes could be produced and achieve a steady state in five to fourteen days.

It is apparent that there are no significant differences between monkeys exposed for 5 days and for 14 days in any of the parameters measured.

These data also indicate that exposure to 220 mg/M^3 CO for 2 week periods does not cause any appreciable change in any of the parameters measured. Previous work in this laboratory showed that

exposure of dogs and monkeys to 550 mg/M^3 CO produced a significant increase in total hemoglobin, hematocrit and red blood cells with an associated rise in reticulocytes reaching a maximum level in approximately four weeks. Although not statistically significant, we did see a uniform rise in total hemoglobin and hematocrit in all monkeys exposed to 220 mg/M^3 CO. The increase could have compensated for any decrease in oxygen content or pO_2.

The monkeys exposed to 440 mg/M^3 did exhibit an appreciable degree of hypoxia as shown by the decrease in oxygen content, saturation and pO_2. It was extremely unfortunate that the blood samples for COHb measurement of this group could not be analyzed. However, repeated studies in this laboratory have shown that COHb levels in monkeys reach a uniform concentration and remain steady over long periods of exposure to constant levels of CO. Smith (1968) has stated that blood COHb reaches equilibrium with inspired CO within 24 hours. It is very likely that the animals in this experiment would have responded in a similar manner and that their COHb levels plateaued at approximately 30%. The degree of hypoxia produced was not sufficient apparently to cause any significant changes in mitochondrial respiratory control or energy production. However, there does appear to be a trend toward a slight decrease in CP with a concommittant increase in ADP, AMP and Pi levels. This would be the expected effect since

CP drops very quickly in response to hypoxia with ATP levels decreasing at a slower rate (McIlwain, 1966; Lowry, 1964). The level of CO required to produce a cellular hypoxia which would affect mitochondrial ATP and CP formation is relatively high.

A ratio of CO to O_2 of about 20:1 is required to produce 50% inhibition of cytochrome oxidases (Keilin et al 1939). However, Omura et al (1965) recently reported that cytochrome P450 shows 50% inhibition when the CO:O ratio is approximately one. Assuming the partial pressure of oxygen at the mitochondria to be 1 mmHg, it is obvious that much higher levels of CO than we employed would be required to achieve a CO:O ratio of 1. We feel that at the exposure levels of CO used in this study the degree of hypoxia produced did not have significant deleterious effects on mitochondrial respiration. The compensatory erythropoetic response was probably sufficient to provide an adequate amount of oxygen to the cells.

In summary, there was no significant change in mitochondrial respiration or energy production in brain tissue from monkeys exposed to 220 mg/M^3 and 440 mg/M^3 CO in an atmosphere of 68% oxygen 32% nitrogen at 5 psia.

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