Hematologic Alterations Produced by Nitrous Oxide

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An atmosphere of 80 vol per cent nitrous oxide and 20 vol per cent oxygen produced erythropoietic and leukopoietic depression in rats in the first two days of a six-day exposure. The depression was rapidly reversed by return to room air. ^{59}Fe and ^{14}C-thymidine distribution studies indicate that hematologic depression occurs at the hematopoietic stem cell or an alternate progenitor cell level. The diminished erythropoietic response to erythropoietin of rats breathing nitrous oxide provides further evidence for this contention. Lymphocytes were relatively resistant to nitrous oxide. Electron spin resonance (ESR) measurements revealed increased free radicals in the liver, whereas levels in the spleen and marrow were too low to measure. Fasting produced similar changes. When animals exposed to nitrous oxide were fasted the free radicals in the liver decreased to levels lower than those in fasted control animals and paralleled the hematopoietic changes. These results suggest that nitrous oxide acts at the level of the hematopoietic stem cell to arrest hematopoiesis, an effect which is rapidly reversible with return to room air. No definite causal relationship between ESR changes and the hematologic changes induced by nitrous oxide could be established. (Key words: Nitrous oxide; Leukopoiesis; Free radicals; Erythropoiesis; Hematopoiesis; Stem cell; Electron spin resonance; Starvation.)

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HEMATOPOIETIC DEPRESSION as a consequence of exposure to nitrous oxide has been observed in experimental and clinical situations.[1-9] The initial observation[1] of granulocytopenia in a patient with tetanus during nitrous oxide therapy has been confirmed by other investigators,[2-4] who also reported thrombocytopenia[2-4] and anemia.[5-7] In addition, marked leukopenia with relative lymphocyte sparing[8] and decreases in hemoglobin, erythrocyte count, and erythroblastic and myeloblastic activity in bone marrow[9] in experimental animals have been reported.

The mechanisms by which nitrous oxide produces hematologic depression has not been clarified. The hematologic abnormalities could result from diminished hematocytogenesis, augmented blood cell destruction or an altered distribution of the peripheral blood cells. These effects either could result from a toxic effect of nitrous oxide or could occur secondary to the associated reduction in oxygen tension.

In the present study, hematopoiesis, with emphasis on the quantitative evaluation of erythropoiesis, was assessed during and following exposure to nitrous oxide. The response to erythropoietic stimulation during exposure was also evaluated. Since a characteristic of nitrous oxide is its ability to function as a free radical scavenger, and since free radicals are important metabolic intermediates in energy transfer systems, the tissue content of free radicals was measured and correlated with the hematologic data.

Methods and Materials

Female Wistar rats, Walter Reed strain, weighing 120–160 g were used. The principles of laboratory animal care recommended by the National Society for Medical Research were observed. Preliminary experiments indi-

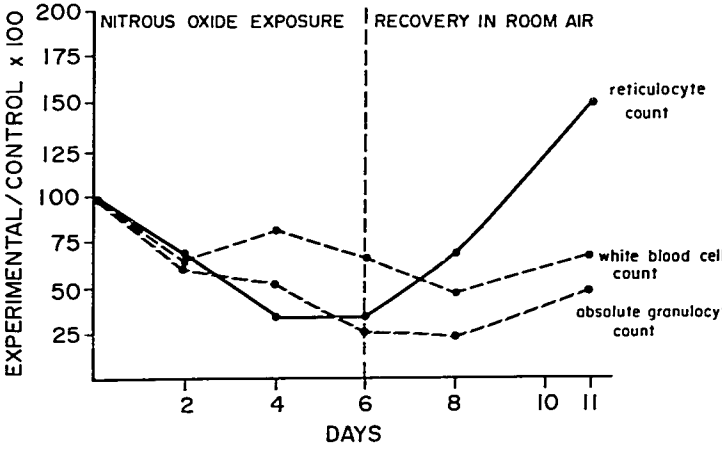

FIG. 1. Peripheral blood measurements. Absolute granulocyte counts were determined by multiplying percentages of granulocytes by total leukocyte counts.

cated that rats exposed to nitrous oxide ate little during the first 24 hours and that food consumption had returned to normal by 48 to 60 hours. Therefore, food given control rats was rationed during the first 48 hours of the experiments, with food § and water allowed *ad lib.* thereafter.

The nitrous oxide atmosphere was provided by means of a plastic isolator and medicinal grade bottled gas mixed to provide 80 vol per cent nitrous oxide and 20 vol per cent oxygen at a flow rate of 4 l/min. Circulation within the isolator was maintained with a Muffin fan and temperature was kept within 3 F of room temperature. Water and carbon dioxide were absorbed by potassium hydroxide and calcium carbonate, respectively. Oxygen and carbon dioxide levels in the exhaust gas and in gas samples obtained at the level of the animal cages in the isolator ranged from 19.5 to 20 vol per cent and from 0 to 2.5 vol per cent, respectively.

The flow rate of bottled compressed air, supplying a similar isolator housing control rats, was adjusted to provide carbon dioxide levels comparable to those in the nitrous oxide isolator. During the recovery period following exposure to nitrous oxide, the rats were kept in their original isolators and a manifold was attached to allow equal flows of compressed air to both isolators.

Groups of four rats each, of approximately the same weight, were placed in either compressed air or nitrous oxide environments. Control and experimental groups of four rats each were killed on the second, fourth, and final (sixth) days of exposure. Control and experimental groups were also sacrificed during the recovery period, eight and 11 days after the experiment began. Duplicate determinations of microchematocrit, hemoglobin, erythrocyte count, reticulocyte count, leukocyte count and differential were made at the time of sacrifice. Eighteen hours after intraperitoneal injection, the distribution in the cells, liver, spleen and marrow of a tracer dose of 1 μCi ^{59}Fe/1 μg ^{56}Fe sulfate was determined [10] as an index of erythropoiesis. The distribution of 1 μCi ^{14}C-thymidine in the blood, spleen, liver and mar-

§ D & G Laboratory Biscuits, D & G Laboratory Animal Food, Frederick, Md.

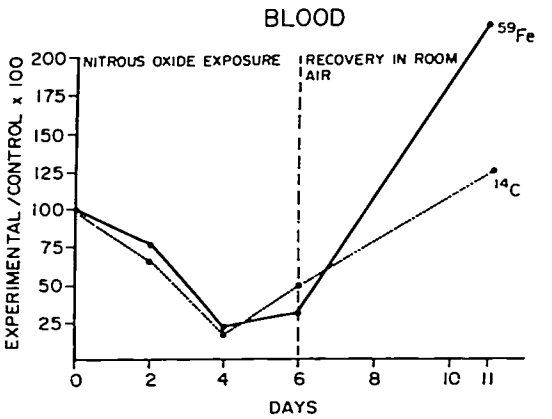

FIG. 2. Distribution of ^{59}Fe and ^{14}C-thymidine in peripheral blood. Each point represents four rats. Measurements at all time intervals were significantly different at the 95 per cent level.

row was measured as an index of nucleic acid synthesis 18 hours after administration.

The erythropoietic response to exogenous erythropoietin was assessed in another experiment. One-ml amounts of anemic rat plasma rich in erythropoietin, prepared by the method of Hodgson and Eskuche,[10] were injected intraperitoneally 48 and 72 hours after the beginning of exposure to nitrous oxide. The 18-hour utilization of radioiron by erythrocytes was determined at 96 hours.

In other experiments, electron spin resonance (ESR) measurements of free radicals were made using livers and kidneys of experimental and control animals. The animals were anesthetized and killed and the samples removed, prepared and analyzed as previously described.[11] Low microwave power (approximately 1 mw) was used to emphasize free radicals. Since nitrous oxide did not change the shapes of the ESR spectra, relative quantitative data were obtained by measurement of the peak–peak heights of the first derivative curves. All samples were run at least twice and samples from at least three animals were averaged to obtain each point. Attempts to measure free radicals in the bone marrow and spleen were unsuccessful because of the low levels of free radicals in these organs. A *t* test for paired data was used to evaluate differences between experimental and control values for each sampling period. $P < 0.05$ was considered significant.

Results

The results of peripheral blood measurements are shown in figure 1. There were no significant changes in hematocrit or hemaglobin concentration in either experimental or control groups at any time. Reticulocyte concentrations were significantly depressed after two, four, and six days of exposure to nitrous oxide, the lowest value being reached by four days (fig. 1). Values increased rapidly during the recovery period and averaged 168 per cent of control levels at the end of the experiment. Granulocyte concentrations progressively decreased during exposure to nitrous oxide and had not returned to control levels at the time of the last sampling (five days post-exposure). Total leukocyte concentrations (81–95 per cent lymphocytes) were less affected than granulocyte concentrations.

Figure 2 shows ^{59}Fe and ^{14}C-thymidine distributions in peripheral blood as a function of time. The ^{59}Fe uptake paralleled the response shown by the reticulocyte count. Both ^{59}Fe uptake and reticulocyte count decreased rapidly through day 4 to about 25 per cent of control values and then remained relatively constant through day 6. At day 11, after five days without N_2O, both values were well

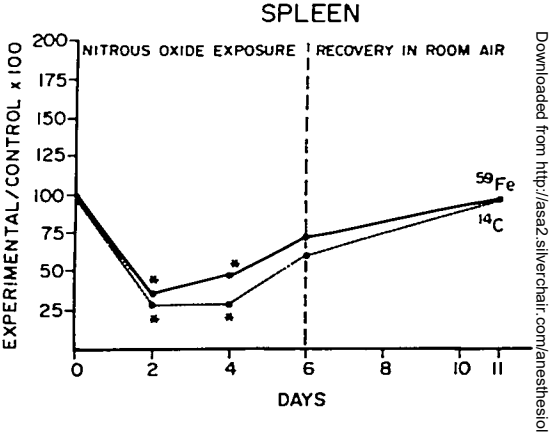

FIG. 3. Distribution of ^{59}Fe and ^{14}C-thymidine in the spleen. * indicates significance at the 0.05 level. Each point represents four rats.

above control levels. The incorporation of ^{14}C-thymidine into peripheral blood cells showed changes similar to those seen in the reticulocyte and granulocyte counts, although lymphocytes, which have low rates of incorporation, comprised 80 per cent of the total leukocytes. At day 11 (five days post-exposure), ^{14}C-thymidine values were significantly elevated compared with controls.

Figure 3 shows comparable isotopic levels in the spleen which functions as a hematopoietic organ in the rat. In contrast to the findings in peripheral blood, incorporations of both ^{59}Fe and ^{14}C had reached their lowest levels by day 2 of exposure. The values returned toward control levels thereafter, and were not significantly different from control values by day 6.

Figure 4 shows the distributions of ^{59}Fe and ^{14}C-thymidine in the bone marrow. Both ex-

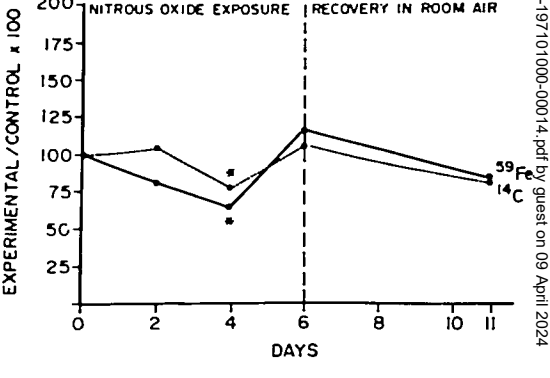

FIG. 4. Distribution of ^{59}Fe and ^{14}C-thymidine in the bone marrow. * indicates significance at the 0.05 level. Each point represents four rats.

TABLE 1. The Erythropoietic Response to Exogenous Erythropoietin

	Experimental Conditions	Number of Rats	Per Cent Reticulocytes	Blood ^{59}Fe (CPM)	Spleen ^{59}Fe (CPM)
Control	96 hours in room air	6	4.4	16,000	16,000
	96 hours in room air + erythropoietin	6	10.1	45,000	17,000
Experimental	96 hours in N_2O	6	1.4	3,000	12,700
	96 hours in N_2O + erythropoietin	6	2.0	8,000	8,000

perimental and control values showed considerable spread, and no significant differences were found except that ^{59}Fe and ^{14}C-thymidine levels had decreased after four days of exposure. Because of this variation, the lack of significant changes does not indicate that these values did not change, but only that in these experiments this measurement was insufficiently precise to reflect changes.

The effects of stimulation with erythropoietin are shown in table 1. Erythropoietin stimulated reticulocyte production and erythrocytic ^{59}Fe uptake two- to threefold in control rats. Erythropoietin also stimulated erythropoiesis in rats exposed to N_2O for 96 hours; however, values for erythrocytic incorporation of ^{59}Fe and reticulocytes did not reach control levels.

Initial experiments indicated (table 2) that by 24 hours the livers of rats kept in nitrous oxide contained increased amounts of free radicals which persisted for at least 72 hours, returning to normal within 48 hours of return to a normal atmosphere. Analysis of the experimental arrangement suggested that these results might have been affected by the decreased food intake of the nitrous oxide-treated animals and, therefore, experiments in which normal animals were denied food were done.

TABLE 2. The Effects of Prolonged Breathing of 80 Per cent N_2O and 20 Per cent O_2 on Free Radicals* in Rat Liver

	Time in Nitrous Oxide		Out 48 Hours
	72 Hours	96 Hours	
Experimental	95 ± 1.3 (4)	101 ± 4.7 (6)	84 ± 2.7 (4)
Control	77 ± 5.0 (4)	73 ± 3.1 (5)	80 ± 6.2 (3)

* Recorded as peak-peak heights of first derivative curves ±SD.

Increases in free radicals similar to those seen in animals exposed to nitrous oxide were found (table 3), suggesting that the initial results could be explained by decreased food intake. A second set of experiments in which both nitrous oxide-treated animals and controls were denied food and water revealed that the level of free radicals in the kidney was unaffected by N_2O, while the level of free radicals in the liver had decreased by four hours and was further depressed after nine hours of treatment (table 4).

Discussion

Nitrous oxide is lethal to the chick embryo,[11] produces cytopathic effects in mammalian cell culture systems,[12, 14] is teratogenic in rodents,[13] and produces hematologic depression in animals and man. The spectrum of effects of prolonged exposure to nitrous oxide ranging from cell death to decreased rates of cellular proliferation has been recognized only recently.[12] Hematopoietic depression was first observed in 1955 by Gorsmen [1] during the course of treatment of a patient with tetanus, when thrombocytopenia and leukopenia were noted. The patient was also receiving chlorpromazine and *d*-tubocurarine and the hematologic alteration was attributed to *d*-tubocurarine. In the same year, Mollaret [2] also found granulocytopenia after nitrous oxide therapy in a patient with tetanus. The following year, Lassen and co-investigators [4] described 13 patients who had tetanus treated with nitrous oxide and attributed their "acute aplastic anemia" to nitrous oxide. Many confirmatory reports of the hematologic toxicity of nitrous oxide in clinical situations followed.[5-7] Subsequently, the hematologic effect of nitrous oxide was utilized in attempts to treat chronic myeloid leu-

kemia.[16, 17] The efficacy of this therapeutic approach remains in doubt. The patients treated in this manner, however, manifested rapid decreases in leukocyte counts, associated with some thrombocytopenia but no change in hematocrit or hemoglobin values.

Studies of the experimental animal and *in vitro* [13, 14, 15] have attempted to clarify the effect of nitrous oxide. Kieler and his coworkers [13] found cytostatic and cytocidal effects of nitrous oxide on myoblasts of mouse hearts in tissue culture. These effects could be correlated closely with the degrees of hypoxia produced, and in addition, could be correlated with nitrous oxide concentrations. Studies by Bruemmer and associates [14] demonstrated decreased multiplication of HeLa cells in monolayer cultures, an effect attributed to interference with cell attachment, perhaps secondary to a change in interfacial pressure between adjacent cell membranes.

Okamoto [9] exposed rats to nitrous oxide and found severe leukopenia with relative lymphocytosis. The hematocrit and erythrocyte count decreased, as did myeloblastic and erythroblastic activity in the bone marrow. Green and Eastwood [8] also studied the effects of nitrous oxide on hematocytogenesis in rats. They found that leukocyte counts decreased to 10 per cent of normal by the sixth day of exposure and that granuocytes disappeared more rapidly from the circulation than lymphocytes. Serial bone marrow examinations revealed progressive hypoplasia, apparent cessation of cell reproduction, disappearance of mitoses. Rapid hematopoietic recovery followed return of the rats to room-air environments.

In the present study the expected decrease in peripheral cellular elements of blood was found. In addition, we were able to determine the sequence of the depression in the peripheral blood elements. Depression was found in the spleen and indications of depression were found in bone marrow before changes in the peripheral blood were evident. Furthermore, the increase in cell genesis with recovery was also found first in the bone marrow and spleen. This suggests that the effect of nitrous oxide on hematopoiesis is exerted on the stem cell or an alternate progenitor cell. The erythropoietic unresponsiveness to physiologic stimulation with erythropoietin further supports the hypothesis that there is a block in cell genesis, probably at the stem cell level. It should be noted that although the rats were maintained in a nitrous oxide environment there was a suggestion of beginning recovery in the spleen and bone marrow before they were returned to an ambient environment. Whether this represents re-equilibration or adaptation to the nitrous oxide-produced hematopoietic block remains unclear.

The results indicate that nitrous oxide depresses hematopoiesis and production of granulocytes sufficiently to account for the clinically observed hematopoietic effects. However, minor effects of cell distribution and/or redistribution are not completely eliminated by these

TABLE 3. The Effects of Fasting on Free Radicals*

Hours without Food	Liver	Kidney
0	102.3	86.1
24	136.9	90.1
48	137.4	91.1
82	136.9	81.1
96	116.9	83.5

* Recorded as peak-peak heights of first derivative curves.

TABLE 4. The Effects of Short-term 80 Per cent N_2O and 20 Per cent O_2 on Free Radicals* in Fasting Rats

	Four-hour Fast		Nine-hour Fast	
	Liver	Kidney	Liver	Kidney
Control	102.3† ± 3.8	74.5 ± 4.2	106.0† ± 4.1	79.0 ± 4.7
Experimental	94.1 ± 2.8	79.1 ± 6.4	84.0 ± 1.3	72.8 ± 2.4

* Recorded as peak-peak heights of first derivative curves ±SD.
† Significant difference from control values (.05 level).

experiments. Equally depressive effects on both leukocyte and erythrocyte production were demonstrated. No effect on the long-lived lymphocytes was evident.

The development of the concept of the electron-pair bond provided the basis for the definition of free radicals. A free radical is any structure containing an atom with an unshared electron or any molecule containing two or more unpaired electrons. A unique property of free radicals is paramagnetism. New developments in techniques and apparatus for electron spin resonance (ESR), which measures paramagnetism, have led to the discovery of numerous free-radical reactions in biological systems. Drugs are a potential source of free radicals in tissues. Phenothiazine drugs have been shown to react with the body by means of free radicals.[19, 20] Interaction of free radicals also apparently is part of the action of imipramine[21] and that of salicylic acid,[22] and ephemeral radicals are present during salicylate oxidation.[23] Conceivably, most, if not all, drugs derive their effects through free-radical mechanisms.

Free radicals are also induced in tissue by radiation. The production of these free radicals is considered a primary mechanism of radiation damage. Indeed, radiation injury may be prevented by the prior administration of compounds such as the aminothiols and their derivatives which are capable of combining with the unpaired electrons; these substances are termed "free-radical scavengers."

Electron spin resonance measurements were undertaken in an attempt to correlate alterations in free radicals with the metabolic changes attendant upon or antedating the diminished hematopoiesis. The reversible hematologic depression produced by ionizing irradiation has been attributed to the generation of free radicals; nitrous oxide has been reported to be a free-radical scavenger.[24] This, together with the findings of Ebert[25] that nitrous oxide protects cells against radiation damage and the demonstration by Evans[26] that nitrous oxide protects the mouse against total-body irradiation, provided impetus to evaluate the changes in ESR spectra. Furthermore, since free radicals are important metabolic intermediates in energy-transfer systems, evaluation would provide some insight into the mechanism of nitrous oxide toxicity. Under the present conditions of measurement, however, no significant alterations in free radicals as determined by ESR spectra were found in the bone marrow or kidney. However, there were serial changes in the liver which were temporally related to the alterations in hematopoiesis in bone marrow and peripheral blood. Since alterations in the free radical content of the liver can result from many causes, no definitive causal relationship to the hematopoietic changes can be established.

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Drugs

DIAZEPAM ON CORONARY FLOW Diazepam (Valium) decreased vascular resistance and increased blood flow in the coronary arteries of dogs. The decrease in vascular resistance was demonstrated by increased flow at a constant perfusion pressure or decreased pressure at a constant flow while the dogs were on cardiopulmonary bypass. By separating the coronary and systemic circulations completely with a double pump-oxygenation system these coronary arterial effects were found to be independent of extracardiac factors. Diazepam produced no changes in myocardial contractility when coronary flow was maintained constant but increased contractility when the flow was allowed to increase. This indicates that changes in coronary flow, and not positive inotropism *per se*, are responsible for the improvement in myocardial function. (*Abel, R. M., Reis, R. L., and Staroscik, R. N.: Coronary Vasodilation Following Diazepam (Valium), Brit. J. Pharmacol. 38: 620 (March) 1970*). ABSTRACTER'S COMMENT: The superiority of diazepam over thiopental as an anesthetic for cardioversion, alleged by many internists, may have some basis in fact if the above results can be extrapolated to man.

HEPARIN AND SERUM LIPOPROTEIN LIPASE When heparin is injected into the circulation it produces a rapid increase in circulating serum lipoprotein lipase. This lipolysis system probably circulates as a heparinapoenzyme complex. Lipoprotein lipase activity disappears exponentially from the circulation. Evidence thus far suggests that a major site of lipoprotein lipase inactivation is the liver. Efficiency of the inactivation system was evaluated in catheterized, unanesthetized dogs by studying the portal vein–hepatic vein lipoprotein lipase activity differential. Results demonstrated the high efficiency of the inactivation system *in vivo* and showed that high levels of heparin may block the inactivation system, suggesting the possibility of a two-step mechanism. The first step in inactivation may involve heparin destruction by hepatic heparinase. This step may induce dissociation of the acute complex. A second step after dissociation may be removal of the apoenzyme. (*Whayne, T. F., Jr., Felts, J. M., and Harris, P. A.: Effect of Heparin on the Inactivation of Serum Lipoprotein Lipase by the Liver in Unanesthetized Dogs, J. Clin. Invest. 48: 1246 (July) 1969.*)