PATHOGENESIS OF ABORTION IN ACUTE NITRITE TOXICOSIS IN GUINEA PIGS

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ABSTRACT

PATHOGENESIS OF ABORTION IN ACUTE NITRITE TOXICOSIS

IN GUINEA PIGS

by Dineshwar P. Sinha

This work was designed to study the effects of acute nitrite toxicosis on reproduction in guinea pigs. Sodium nitrite was used as the source of nitrite.

Since formation of methemoglobin is the major factor in the pathogenesis of nitrite toxicosis, methemoglobinization of blood *in vitro* was studied and the data obtained were used to build a model for the *in vivo* studies.

The lethal dose of $NaNO_2$ for guinea pigs was determined. Methemoglobin and plasma nitrite levels after administration of $NaNO_2$ were also determined. The pathologic and embryotoxic effects of acute $NaNO_2$ toxicosis at various stages of gestation were studied. A postmortem examination of each animal was performed and sections of various tissues were prepared for histopathologic examination. The protective effect of methylene blue on $NaNO_2$ toxicosis was evaluated. Maternal and fetal P_{O_2}, P_{CO_2} and blood pH values were determined after administration of $NaNO_2$ to the dam.

Methemoglobinization occurred at a faster rate and higher levels were attained *in vitro* with fetal blood than with maternal blood. However, the reduction of methemoglobin to hemoglobin was faster in fetal than in maternal blood. After parturition the differences in methemoglobinization

between maternal blood and blood from the newborn were reduced considerably.

Studies in vitro indicated that even in the case of a high degree of methemoglobinization, the enzyme system was capable of reducing methemoglobin to hemoglobin.

Following subcutaneous administration, $NaNO_2$ was absorbed rapidly and the plasma nitrite level reached a peak between 7.5 and 15 minutes. The highest level of methemoglobin was observed about an hour after $NaNO_2$ administration.

When dams were given $NaNO_2$ subcutaneously, maternal plasma nitrite values were higher than fetal plasma nitrite values, indicating that there may be a partial placental barrier to transport of nitrite to the fetus.

In the last quarter of pregnancy guinea pigs given $NaNO_2$, 50 mg./kg., underwent normal parturition, whereas in those given $NaNO_2$, 60 mg./kg., fetal mortality occurred and was followed by abortion 1 to 4 days after treatment. The fetal deaths occurred when the maternal and fetal methemoglobin levels were at their peak. At the time of fetal death there were no significant changes in the placenta; pathologic changes developed after death of the fetuses. The necrotic changes observed in the placentas were therefore a consequence rather than a cause of the fetal deaths.

In pregnant guinea pigs given $NaNO_2$, 60 mg./kg., and treated simultaneously with methylene blue, 10 mg./kg., no fetal deaths occurred.

There were lower P_{O_2} and higher P_{CO_2} values in the fetuses of the guinea pigs treated with $NaNO_2$, 60 mg./kg., than in fetuses of the control animals. The results strongly suggest that fetal deaths resulted from hypoxia induced by methemoglobinemia.

Dineshwar P. Sinha

The administration of $NaNO_2$, 60 mg./kg., to guinea pigs in the first half of pregnancy produced less fetal mortality than when given in the last quarter of pregnancy. When $NaNO_2$, 60 mg./kg., was given to guinea pigs on or about the 30th day of pregnancy, deformity of the hind leg was observed in 2 of 16 newborn guinea pigs.

PATHOGENESIS OF ABORTION IN ACUTE NITRITE TOXICOSIS IN GUINEA PIGS

By

Dineshwar Prasad Sinha

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Many persons have contributed significantly to the development of this work. To them I wish to express my gratitude, not only for their influence on my academic activities, but also for the part they have played in shaping my personal life during this period. The brevity of this section by no means reflects the nature of my recognition of their importance, which I believe will be lifelong.

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INTRODUCTION

Nitrite poisoning among animals and man has been observed for centuries and reported from time to time as different syndromes by various observers.

With the advancement of technology, the use of nitrite is increasing as a food preservative, drug, herbicide and fertilizer. This may lead to various health hazards, including abortion and possible teratogenic effects.

Cases of abortion have been observed with nitrite poisoning in animals. The purpose of this experiment was to elucidate the pathogenesis of abortion in acute nitrite poisoning. Sodium nitrite was used in all the experiments to eliminate variables which might be introduced by using different nitrite compounds.

The guinea pig was selected as the test animal for reasons of economy, litter size, size of fetuses and because the animal is easy to work with.

Nitrite reacts with hemoglobin and forms methemoglobin. Methemoglobin leads to hypoxia or anoxia depending upon the level of methemoglobin present in the circulating blood. Hypoxia produces teratogenic effects, so it was important to study the effects of nitrite early in gestation when teratogenic effects would be most likely.

REVIEW OF LITERATURE

Nitrate and Nitrite Intoxication

Nitrate fertilizer is palatable, especially to cattle, and has caused poisoning. Plants containing 2% or more of nitrate (dry weight) are dangerous to feed and may cause poisoning. In nitrate poisoning the major toxicant is nitrite which has been formed by the reduction of nitrate, probably by bacterial or enzymatic action within the digestive tract of the animal (Binns, 1956). The reduction of ingested nitrate in the digestive tract occurs readily in ruminants and other herbivorous animals. This is not the case in omnivorous and carnivorous species. However, all species are susceptible to poisoning by dietary nitrites (Jones, 1965).

Riggs (1945) reported that nitrate as such is relatively nontoxic. The toxicity is proportional to the amount of nitrate reduced to nitrite, which is readily absorbed from the digestive tract. Nitrite combines with hemoglobin to form methemoglobin, which produces signs similar to cyanide poisoning. His experiment, conducted with oat hay and turkey feed moistened with distilled water, showed that approximately half of the nitrate was reduced to nitrite and was present as such after 20 hours. He concluded that this reduction to nitrite may account for the poisoning associated with the ingestion of high nitrate hays following rains.

McIlwain and Schipper (1963) reported that diets containing more than 0.5% potassium nitrate are toxic. Acute signs of intoxication are

related to an interference with oxygen-carrying elements of the blood. Chronically a vitamin A deficiency may occur possibly due to digestive irritation that results in decreased conversion of carotene. Goodrich (1962) reported that sheep fed 3% sodium nitrate in rations with or without the addition of vitamin A had significantly lower hepatic vitamin A stores.

Muhrer et al. (1956) observed signs of vitamin A deficiency in cattle that had consumed nitrate. Garner et al. (1958) reported increased depletion of vitamin A in rats fed nitrate. Flynn (1961) found that hepatic vitamin A levels were low in sheep fed nitrate (0.1% to 0.75%).

O'Dell (1960) mentioned that nitrite caused depletion of vitamin A, and also precipitated a vitamin E deficiency in rats on a diet normally adequate in vitamin E (5 mg./100 Gm.). Further evidence of the vitamin E deficiency was shown by the improvement of appetite and muscular coordination when vitamin E concentrate was fed to the rats.

It has been suggested that the nitrate ion may serve as a thyroid depressant by interfering with iodine metabolism (McIlwain and Schipper, 1963). Welsch (1961) reported enlarged thyroid glands in rats consuming a diet containing 2.5% KNO_3. Experimental results indicated that 0.31% and 0.92% dietary nitrate, consumed by rats and sheep, respectively, can affect the normal iodine metabolism of the thyroid gland (Bloomfield et al., 1961). The function of the thyroid gland in the conversion of carotene to vitamin A is still debated. However, Johnson and Baumann (1947) showed that a functioning thyroid gland is necessary for this conversion.

Nitrites in the body lower the tonus of arterial muscles producing a prompt fall in blood pressure. This action is directly on the smooth muscles and is mutually antagonistic with epinephrine (Sollmann, 1957).

Naider and Venkatrao (1945) reported 30 cases of fatal nitrite poisoning in man. They estimated the lethal dose for an adult to be about 2 Gm. of sodium nitrite or about 2.6 Gm. of potassium nitrite. Simon et al. (1959) stated that a lethal dose of potassium nitrate for cattle is 25 Gm./100 lb. of body weight.

Case (1957) concluded that signs of nitrate intoxication vary from abortion and decreased production to severe intoxication that soon ends in death. The severity depended on the excess (over 0.5%) of nitrate in the rations. Cattle and sheep fed high quality rations could tolerate more (1.5%) nitrate in the total ration, whereas cattle on poor or deficient rations died with less (0.7%). Addition of carbohydrate and vitamin A helped to reduce the toxicity if the nitrate content of the total ration was not over 1.5%.

Methemoglobinemia Produced by Nitrites

Nitrites, as oxidizing agents, react with hemoglobin to form methemoglobin; this decreases the nitrite content of the plasma. The oxidation continues as long as the nitrite ions are in contact with the red blood cells. When nitrite-containing plasma or serum is separated from the red blood cells, the reaction ceases (Marich, 1965).

Methemoglobin, also known as hemiglobin and ferriglobin, is a derivative of hemoglobin in which the ferrous porphyrin complex is converted to the ferric form which does not combine with oxygen and is therefore of no value in respiration. The presence of the reducing enzymes in the

blood prevents complete oxidation of hemoglobin to methemoglobin. These enzymes are known as diaphorase I and II. The cofactor for diaphorase I is diphosphopyridine nucleotide (DPN), and triphosphopyridine nucleotide (TPN) is the cofactor for diaphorase II. In nitrite poisoning, the nitrite ion is a sufficiently good oxidizer to overcome the normal reducing effects of the diaphorase enzymes on methemoglobin. The nitrate ion does not have this effect (Jones, 1965). Austin and Drabkin (1935) noted that 0.5 to 0.7 mole of sodium nitrite is effective in converting 1 mole of oxyhemoglobin to methemoglobin. The alteration of the shape and position of the oxygen dissociation curve in methemoglobinemia implies that the tissues are liable to anoxia, not only because of loss of the oxygen carrying capacity of the blood, but also because the residual oxyhemoglobin is less capable of dissociating and thereby releasing oxygen in the tissues (Bodansky, 1951).

Methemoglobin has a characteristic light absorption at 635 mμ. This absorption is abolished in the presence of cyanide, which converts methemoglobin to cyanmethemoglobin. The difference in light absorption at 635 mμ. before and after adding cyanide is a measure of the methemoglobin present (Evelyn and Malloy, 1938).

Winter (1962) reported that hydroxylamine was formed from nitrite in the blood and was itself capable of converting hemoglobin to methemoglobin. Treatment with nitrate or nitrite had little effect on blood ammonia levels.

Abbanat and Smith (1964) induced methemoglobinemia in female mice by the intraperitoneal injection of sodium nitrite (75 mg./kg.). The peak methemoglobin formation (34%) was achieved in about 40 minutes.

Keohane and Metcalf (1960) stated that there are gradual changes in the sensitivity of erythrocytes to sodium nitrite during childhood with a rapid increase in resistance at about the time of puberty. Hemoglobin of cord blood is more susceptible to methemoglobinization by sodium nitrite solution than adult hemoglobin.

Ross (1963) reported that the blood of premature infants generally has a higher methemoglobin concentration than does that of infants born at term, and that they are more susceptible to the development of methemoglobinemia upon exposure to aniline dye. The TPN dependent methemoglobin reductase is decreased in cord blood.

Emerick et al. (1965) disclosed that pigs, given an intravenous injection of 0.03 Gm. sodium nitrite (2%) per kg. body weight had a lower degree of methemoglobinemia when treated at 1 week of age (32%) than the same pigs when similarly treated at approximately 3 months (69%) and 5.5 months (80%) of age.

Studies by Stolk and Smith (1966) revealed that the hemoglobin of man, mouse, and rabbit show equivalent sensitivity to oxidation by sodium nitrite. The greater resistance of certain species to methemoglobin producing agents is due to higher levels of methemoglobin reductase activity. It is likely that the cat metabolizes aromatic amino compounds to methemoglobin-forming intermediates more actively than the other species.

Setchell and Williams (1962) observed that after oral administration of sodium or potassium nitrate to sheep (nitrate equivalent to 2% of the food intake) the methemoglobin conversion reached a maximum of 40 to 65% of the hemoglobin. The maximum usually occurred 4 hours after drenching and then slowly declined. Repeated drenching for 6 days had

no cumulative effect on the concentration of nitrate or nitrite nitrogen of the plasma or the level of methemoglobin formation.

Effect of Nitrite on Reproduction

Sund and Wright (1957) reported several cases of abortion in cattle due to grazing on weeds of high nitrate content. The placentas of the aborted fetuses had degeneration of the cotyledons with numerous focal areas of mineral deposits. Garner et al. (1958) fed 4 lots of sows, 2 sows in each lot (starting 35 days after breeding), a ration containing 0.0%, 0.5%, 1.0%, and 2.0% of potassium nitrate. Serum nitrate levels were elevated with increasing amounts of nitrate in the ration. They mentioned that the litter size was not affected, but viability and the number of strong pigs decreased with the higher levels of the nitrate.

Simon et al. (1958) reported that in the lowland abortion syndrome in Wisconsin the characteristic pathologic changes included perirenal hemorrhage and marked kidney degeneration in the fetus, numerous circumscribed, calcified necrotic lesions in the intercotyledonary areas of the fetal membranes, and pleural thickening and vascular changes in the lungs of the dam and aborted fetuses. All pathologic changes observed, including the abortion, could be attributed to anoxia. They found that abortions were associated with grazing on weeds.

Simon et al. (1959) placed KNO_3 (100 Gm.) in the rumen of pregnant dairy cattle. The blood of 1 heifer had 44.2% methemoglobin at the time of abortion. Another heifer had 65.5% methemoglobin in the blood and 2 days later aborted. On the day of abortion the methemoglobin value was 8.62%.

However, Winter (1964) stated that heifers given a balanced ration can maintain normal pregnancies despite prolonged ingestion of nitrate

or nitrite sufficient to convert 40 to 50% or more of the hemoglobin to methemoglobin.

Muhrer et al. (1956) concluded that a 1.0% to 2.0% concentration of potassium nitrate in the ration of the female rat interfered with normal reproduction.

When female guinea pigs were given sodium nitrite in drinking water (0.5% or more), abortion, absorption or mummification of fetuses occurred (Sleight and Atallah, 1968).

Hypoxia and Teratogenic Effects

Since nitrite poisoning leads to hypoxia or anoxia, it was important to consider the effect of hypoxia on fetuses.

Oxygen deprivation, if complete, causes immediate death of the fetuses and, if partial, produces detrimental changes, the degree of which depends on the duration and severity of the lack of oxygen (Potter and Adair, 1949).

A study on the effects of moderate hypoxia on chick embryos has shown that many of the induced malformations are caused by extensive edema followed by the formation of clear blisters and hematomas. The malformations have been attributed to a disturbance in fluid balance (Grabowski, 1961a).

Grabowski (1961b) exposed 2000 three-day-old chick embryos to an environment containing from 0 to 21% oxygen, at normal pressure for periods of 3 to 24 hours. Abnormal development was initially obtained after exposures of 6 to 12 hours at an oxygen level of 13%, whereas the greatest maldevelopment (in up to 40% of the embryos) occurred at about 5% oxygen. There was no maldevelopment at 2% oxygen because of high death rate.

A considerable variety of anomalies were detected in chick embryos (18 hours to 9 days of age) treated for 6 hours with less than 60% of normal oxygen concentrations. These abnormalities ranged from very slight defects to those making the embryo a complete monstrosity. A progressively increasing sensitivity to the teratogenic effect of hypoxia with advancing age was noted (Grabowski and Paar, 1958).

MATERIALS AND METHODS

Technics, Chemicals and Care and Management of the Animals

Bleeding. To obtain large quantities (> 2 ml.) of blood, cardiac punctures were performed on anesthetized animals. The puncture site was swabbed with 70% alcohol. A 1.5 inch, 20-gauge needle was inserted lateral to the sternum and aimed toward the point of the heart beat. When smaller amounts (< 2 ml.) of blood were required, the bleeding was done by incising the paw. Whenever this procedure was employed, the feet were washed and swabbed with 70% alcohol. Hemostasis was accomplished with tannic acid and a bandage was applied.

Anticoagulant. Heparin* was used as the anticoagulant (approximately 1 drop from a 25-gauge needle for 5 ml. of blood).

Determination of Methemoglobin (MetHb.). Methemoglobin values were determined photometrically using the Evelyn and Malloy (1938) procedure. As soon as possible the blood was mixed in M/60 phosphate buffer** in order to prevent reduction of the methemoglobin (Sleight and Sinha, 1968).

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* Heparin (Ammonium Salt) - Biological Research, Inc., St. Louis, Mo.

** A M/15 stock phosphate buffer (pH 6.6) was prepared by dissolving 9.0 Gm. of $Na_2HPO_4 \cdot 12H_2O$ and 5.7 Gm. of anhydrous KH_2PO_4 in water and diluting to 1 liter. Three volumes of water were added to 1 volume of the stock buffer to prepare M/60 buffer.

Determination of Plasma Nitrite. The blood was centrifuged soon after collection and the plasma was removed. The procedure of Diven et al. (1962) was used. The readings were taken on a Coleman Junior Spectrophotometer.*

Sodium Nitrite. For all experiments a fresh solution of sodium nitrite ($NaNO_2$)** was prepared and used within an hour.

Care and Management of the Guinea Pigs. They were housed in 30 x 18 x 12 inch steel boxes. Pelleted Rockland guinea pig diet*** and water were provided ad libitum.

Methemoglobinization of Guinea Pig Blood Induced by $NaNO_2$ (in vitro)

Determination of Effect of Different Concentrations of $NaNO_2$. Blood samples were collected from 4-month-old male guinea pigs by cardiac puncture. Each guinea pig was used only once. Test tubes (15 x 125 mm.) were placed in a 37 C. water bath, and 5 ml. of the heparinized blood was added to each tube. Sodium nitrite solution (1%) was added to the blood so that the tubes contained the equivalent of 5 mg., 10 mg., 30 mg., 40 mg., 80 mg., and 100 mg. per 100 ml. of blood, respectively. During the test, the tubes were slowly rotated in the water bath in order to keep the red blood cells in suspension. Hemoglobin, methemoglobin and packed cell volume (PCV) were determined at specific intervals for each sample.

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* Coleman model 6D Junior Spectrophotometer - Coleman Instruments, Inc., 42 Madison Street, Maywood, Ill.

** J. T. Baker Chemical Co., Phillipsburg, N.J.

*** Teklad, Inc., Monmouth, Ill.

Determination of Effect of Dilution and Plasma Removal on in vitro Methemoglobinization of Blood by $NaNO_2$. A heparinized sample of blood was collected from a guinea pig by cardiac puncture. The specimen was then divided into 3 parts as follows: 5 ml. of whole blood were transferred to the first test tube; for the second tube 5 ml. of blood were centrifuged, the plasma removed and the cells washed in physiological saline (0.85%) and centrifuged again; the supernatant was discarded, the cells resuspended in physiological saline and the volume brought to 5 ml.; 2.5 ml. of whole blood were added to 2.5 ml. of plasma from the same animal for the third tube.

Two-tenths milliliter of 1% $NaNO_2$ solution was added to all 3 test tubes so that each contained the equivalent of 40 mg./100 ml. This experiment was repeated.

Determining Influence of Age on Methemoglobinization. Blood was obtained from guinea pigs of the following ages: 1, 3, and 11 days, 3 and 9 weeks, and 4.5 months. Sodium nitrite solution was added to each tube so that the blood contained the equivalent of 40 mg./100 ml. of $NaNO_2$. Similar studies were also made on fetal and maternal blood samples.

Reduction of High Levels of Methemoglobin to Hemoglobin in vitro. Sodium nitrite solution (1%) was added to 5 ml. of heparinized guinea pig blood so that it contained the equivalent of 80 mg./100 ml. The tube was placed in a water bath at 37 C. Hemoglobin and methemoglobin levels were determined on the sample at 38 and 68 minutes after addition of the $NaNO_2$. Next the blood was centrifuged, plasma removed and the cells were washed 3 times with physiological saline to remove

extraerythrocytic nitrite. Following the last wash the supernatant was discarded and the cells were resuspended in the guinea pig's plasma; the volume was brought to 5 ml. The tube was placed back into the 37 C. water bath and the hemoglobin, methemoglobin and PCV values were determined at various intervals.

Determination of Lethal Doses of $NaNO_2$ for Guinea Pigs

Males. A freshly prepared 2% $NaNO_2$ solution was given subcutaneously as follows: 100 mg./kg. to 10 guinea pigs, 90 mg./kg. to 10 guinea pigs, 80 mg./kg. to 10 guinea pigs, 70 mg./kg. to 10 guinea pigs and 60 mg./kg. body weight to 10 guinea pigs. The LD_{50} was estimated from the mortality data by using logarithms of the doses plotted against the percentage of mortality on a particular scale (DuBois and Geiling, 1959).

Pregnant Females. Sodium nitrite was given subcutaneosuly to 36 pregnant guinea pigs at the rate of 60 mg./kg. body weight. Four other pregnant guinea pigs were given a subcutaneous injection of $NaNO_2$ at the rate of 70 mg./kg. body weight.

Fetuses. Three pregnant guinea pigs in the last quarter of gestation were used for this study. The guinea pigs were anesthetized by giving pentobarbital sodium* (28 mg./kg.) and laparotomies were performed. The number of fetuses was counted and the approximate weight of each fetus was estimated. Varying doses of $NaNO_2$ were administered subcutaneously to the fetuses. At least 1 fetus in each litter was left untreated. The incision was closed and an hour after treatment the

* Pentobarbital Sodium Solution - Haver-Lockhart Laboratories, Kansas City, Mo.

fetuses were examined. At that time the fetuses were weighed and the exact amount of $NaNO_2$ given on a body weight basis was calculated.

Determination of Levels of Methemoglobin and Plasma Nitrite After Administration of $NaNO_2$ to Guinea Pigs

Different Doses of $NaNO_2$ to Male Guinea Pigs. A specific amount of $NaNO_2$ (10, 20, 30, 40, 60, 70 or 80 mg./kg. body weight) was injected subcutaneously. At timed intervals blood was drawn; hemoglobin and methemoglobin values were determined. Plasma nitrite was also determined in guinea pigs given 40, 60 or 80 mg./kg. of $NaNO_2$.

Evaluation of Protective Effect of Methylene Blue on $NaNO_2$ Toxicosis. Methylene blue (1%) was administered intraperitoneally to a guinea pig at the rate of 40 mg./kg. body weight. Two guinea pigs received 60 mg./kg. body weight of $NaNO_2$ subcutaneously and 1 of these guinea pigs was also given 40 mg./kg. of methylene blue (1%) intraperitoneally. By incising the paw, blood samples were taken from both guinea pigs before injection and 1, 2 and 3 hours after administration of the chemicals. Hemoglobin and methemoglobin levels were determined on each sample. The same experiment was done by using 100 mg./kg. body weight of $NaNO_2$ and 10 mg./kg. body weight of methylene blue. This time methemoglobin and hemoglobin were determined before and 75 minutes after administration of the chemicals.

Determination of Methemoglobin and Plasma Nitrite in Maternal and Fetal Blood During Last Quarter of Pregnancy. Sodium nitrite (40 mg. or 60 mg./kg.) was administered subcutaneously to pregnant guinea pigs. Blood samples were taken at intervals of 10, 20, 40 and 60 minutes after

injection for the determination of hemoglobin and methemoglobin. Blood was obtained by incising the paw and each toe was used only once.

Four pregnant guinea pigs were given $NaNO_2$ subcutaneously at the rate of 40 mg./kg. body weight and a hysterotomy was performed on 1 each at 20, 40, 60 and 80 minutes after $NaNO_2$ administration. Ether was used as the anesthetic. Fetal blood was collected by cutting the umbilical cord and maternal blood was obtained by incising the paw. Levels of hemoglobin, methemoglobin and plasma nitrite were determined for each blood sample.

Five pregnant guinea pigs were each given a subcutaneous injection of $NaNO_2$ at the rate of 60 mg./kg. body weight. A similar method as described above was used to obtain maternal and fetal blood at intervals of 20, 60, 100 and 140 minutes after injection. Hemoglobin, methemoglobin and plasma nitrite were determined on maternal and fetal blood. For controls, similar procedures were performed on normal untreated pregnant guinea pigs.

To follow maternal and fetal blood values in the same animal a technic was devised to take repeated samples of maternal and fetal blood at intervals. Pregnant guinea pigs were each given a subcutaneous injection of $NaNO_2$ (60 mg./kg.) and after 15 minutes an anesthetic, pentobarbital sodium (28 mg./kg.), was given. Laparotomy was then performed. Hemorrhages were controlled by ligation and clamping of the blood vessels. An incision was made in the uterine wall, 1 fetus was taken out, and the incision was closed. Fetal blood was collected by cutting the umbilical cord. At the same time maternal blood was collected by incising the paw. Manipulation was kept to a minimum in order to avoid any physical interference with blood circulation. Gauze soaked in physiological

saline was placed over the exposed tissues to avoid dehydration. In a similar way, maternal and fetal blood samples were taken at 25, 40 and 60 minutes after administration of the $NaNO_2$.

Pathologic and Embryotoxic Effects of Acute $NaNO_2$ Toxicosis in the Last Quarter of Pregnancy

Experiment 1. Six pregnant guinea pigs were divided into 3 groups of 2 each, and each group was placed in a separate box. Two groups were given subcutaneous injections of $NaNO_2$ (2%) at the rate of 50 mg./kg. body weight. One group, used as controls, was each given a subcutaneous injection of 2% NaCl at the rate of 50 mg./kg. body weight. The rectal temperature was recorded before and 0.5, 1, 2, and 4 hours after administration of the $NaNO_2$. Reproductive performance was recorded.

Experiment 2. Two groups of 2 pregnant guinea pigs each were used. One group was each given a subcutaneous injection of $NaNO_2$ at the rate of 50 mg./kg. body weight. The other group served as controls, and each was given a subcutaneous injection of NaCl (2%) at the rate of 50 mg./kg. body weight. An experimental and control animal each were killed at 12 and 24 hours, respectively, after administration of the chemicals. Blood samples were collected before injection of the chemicals and just before killing. Determinations for hemoglobin and methemoglobin were done on each blood sample. At the time of necropsy samples of adrenal, brain, heart, intestine, kidney, liver, lung, pancreas, placenta, spleen and uterus were fixed in 10% buffered formalin or in Zenker's fluid. Paraffin sections were made and stained with hematoxylin and eosin. Formalin-fixed sections of liver were also stained with Oil Red O stain.

Experiment 3. Six guinea pigs were divided at random into 3 groups of 2 each. Two groups were given $NaNO_2$ (2%) subcutaneously at the rate of 60 mg./kg. body weight. One group received NaCl (2%) subcutaneously at the rate of 60 mg./kg. body weight. Reproductive performances were recorded.

Experiment 4. Twenty pregnant guinea pigs were divided at random into 10 groups of 2 each and each group was housed in a separate box. Hemoglobin, methemoglobin, total and differential leukocyte counts, PCV, and plasma nitrite were determined on all the guinea pigs before treatment. In 5 groups $NaNO_2$ solution (2%) was injected subcutaneously at the rate of 60 mg./kg. body weight. In the other 5 groups NaCl solution (2%) was injected subcutaneosuly at the rate of 60 mg./kg. body weight to serve as controls. One experimental and one control guinea pig each were killed at the following intervals after administration of the $NaNO_2$ or NaCl: 0.25, 1.5, 3, 6, 12, 18, 24, 24.5, 48 and 56 hours. The guinea pigs were given a general anesthetic (ether) before killing. Hysterotomies were performed and the fetuses removed. Fetal and maternal blood samples were collected. The fetuses were examined to see if they were dead or abnormal. From the maternal blood, hemoglobin, total and differential leukocyte counts, PCV's, and methemoglobin and plasma nitrite levels were determined. From the fetal blood, hemoglobin, methemoglobin and plasma nitrite levels were determined. Blood samples were stained with 1% new methylene blue and examined for the presence of Heinz bodies. Tissues from adrenal, brain, heart, intestine, kidney, liver, lung, pancreas, placenta, spleen and uterus were fixed in 10% buffered formalin or in Zenker's fluid. Paraffin sections were made and stained

with hematoxylin and eosin. Formalin-fixed sections of liver were also stained with Oil Red O stain.

Prevention of Acute Toxicity of $NaNO_2$ in Pregnant Guinea Pigs by Treatment with Methylene Blue

Group 1. In 6 pregnant guinea pigs (last quarter of pregnancy) methylene blue was administered intraperitoneally at the rate of 10 mg./kg. body weight, and in 3 of these animals $NaNO_2$ was given subcutaneously at the rate of 60 mg./kg. body weight. One nitrite-treated animal and 1 given only methylene blue were anesthetized with ether and hysterotomies were performed at the following intervals, after administration of chemicals: 3, 24 and 100 hours.

Group 2. Six pregnant guinea pigs of the same age and approximately in the last quarter of pregnancy were selected for this experiment. In 2 guinea pigs methylene blue was injected intraperitoneally at the rate of 10 mg./kg. and $NaNO_2$ at the rate of 60 mg./kg. body weight. Two guinea pigs were given only methylene blue intraperitoneally at the rate of 10 mg./kg. body weight. The 2 other guinea pigs were given only $NaNO_2$ subcutaneously at the rate of 60 mg./kg. body weight. Reproductive performances were recorded.

Effects of $NaNO_2$ Administration on Pregnant Guinea Pigs During First 30 Days of Gestation

Group 1. Six pregnant guinea pigs at about 10 days of gestation were used. Four guinea pigs were given $NaNO_2$ subcutaneously at the rate of 60 mg./kg. body weight. Two guinea pigs were given NaCl subcutaneously

at the rate of 60 mg./kg. body weight and served as controls. One nitrite-injected animal was killed on the 7th day and 1 on the 14th day after administration of the nitrite. Two nitrite-injected animals and 2 controls were allowed to complete gestation.

Group 2. Nine pregnant guinea pigs at about the 20th day of gestation were selected. Six animals were given $NaNO_2$ subcutaneously at the rate of 60 mg./kg. body weight. Three guinea pigs were given NaCl subcutaneously at the rate of 60 mg./kg. body weight and served as controls. One experimental animal was killed at each of the following intervals after the start of the experiment: 2nd, 3rd, 8th and 11th day. One control animal was killed on the 2nd and another on the 8th day of the experiment. Two experimental animals and 1 control were kept under observation and allowed to proceed to term.

Group 3. Nine pregnant guinea pigs at approximately the 30th day of gestation were used. Six were given $NaNO_2$ subcutaneously at the rate of 60 mg./kg. body weight. The 3 controls were given NaCl (2%) subcutaneously at the rate of 60 mg./kg. body weight. One of the sodium nitrite-injected animals was killed on the 2nd, another on the 7th, and a third on the 14th day of the experiment. One of the controls was killed on the 7th day. Two experimental and 2 control animals were not killed but were kept under observation.

Determination of Maternal and Fetal Blood P_{O_2}, P_{CO_2}, pH and Methemoglobin Values after Administration of Sodium Nitrite

For this experiment 9 pregnant guinea pigs in the last week of gestation were used. Three were injected subcutaneously with $NaNO_2$ at the

rate of 60 mg./kg. body weight. Blood samples were then collected at 30, 60 and 90 minutes after the injection. Maternal blood was drawn from uterine blood vessels and the fetal blood from umbilical blood vessels.

Three of the pregnant guinea pigs were given $NaNO_2$ subcutaneously at the rate of 45 mg./kg. body weight and maternal and fetal blood samples were collected at 30, 60 and 90 minutes after administration.

Maternal and fetal blood samples were also drawn from 3 untreated guinea pigs, to determine normal values.

Immediately after the blood was obtained a Radiometer* was used to determine the P_{O_2}, P_{CO_2} and pH.

* Radiometer A/S - 72, Emdrupvej, Copenhagennv, Denmark.

RESULTS

Methemoglobinization of Guinea Pig Blood by $NaNO_2$ (in vitro)

Effect of Different Concentrations of $NaNO_2$. The methemoglobin level in blood containing $NaNO_2$ (5 mg./100 ml.) reached a peak of 12.5% after 30 minutes and returned to less than 1% by the end of 2.5 hours. With increasing concentrations of $NaNO_2$ there was a tendency towards prolongation of the time before attaining the methemoglobin peak. Also the time for reduction of methemoglobin was prolonged and incomplete at the higher levels of $NaNO_2$ (Table 1). There was no appreciable variation in the PCV values at the various intervals for each blood sample.

Effect of Dilution and Plasma Removal on in vitro Methemoglobinization of Blood by $NaNO_2$. The whole blood, after addition of $NaNO_2$, had a methemoglobin peak of 86.4% at 1.5 hours, which was gradually reduced to 11.8% at 5 hours.

Methemoglobin levels in the samples of RBC's suspended in physiological saline were progressively elevated with time and little if any reduction had taken place by 5 hours (Figure 1).

Blood diluted 50% with plasma also reached a methemoglobin peak, this time 89%, after 1.5 hours. By the 5th hour the methemoglobin level was 53%.

Effect of Age of the Animal on Methemoglobinization. Fetal blood underwent methemoglobinization at a faster rate and higher levels were

Table 1. *In vitro* methemoglobinization of guinea pig blood induced by $NaNO_2$

$NaNO_2$ (mg./100 ml.)	Methemoglobin Level (%) at Intervals												
	0 min.	5 min.	15 min.	30 min.	45 min.	1 hr.	1.5 hrs.	2 hrs.	2.5 hrs.	3 hrs.	4 hrs.	5 hrs.	12 hrs.
5	0.0	0.0	4.1	12.5	5.7	4.4	1.6	1.2	0.5	0.9	0	0	---
10	0.0	1.4	9.7	13.2	13.5	11.1	4.7	0.9	1.2	2.4	0	0	---
30	0.0	---	22.2	31.9	---	25.3	16.4	4.2	---	0.2	1.3	0	---
40	0.4	49.7	55.4	---	---	69.0	56.7	---	43.3	---	4.3	1.3	0.0
80	0.0	20.0	33.8	44.1	45.8	58.8	50.7	74.6	77.7	83.6	66.4	61.4	6.8
100	0.0	35.0	36.8	51.5	60.3	67.6	72.5	88.6	89.9	89.9	80.0	65.7	29.9

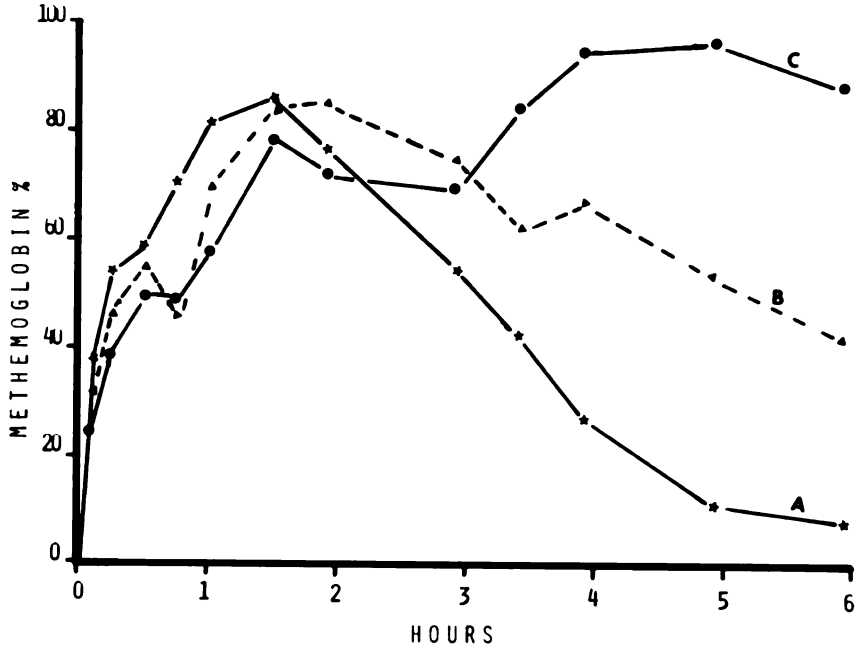

Figure 1. Effect of $NaNO_2$ (40 mg./100 ml.) on whole blood (A), blood diluted 50% in plasma (B), and RBC's suspended in physiological saline (C).

attained than with maternal blood. Also, the reduction of methemoglobin to hemoglobin was quicker than in maternal blood (Figure 2). These differences were reduced considerably after parturition. Three days following birth only a minimal difference was noted. Only slight differences in the rate of formation or reduction of methemoglobin could be detected in the blood from guinea pigs 9 weeks of age or older (Table 2).

Reduction of High Levels of Methemoglobin to Hemoglobin (in vitro). After the addition of $NaNO_2$ to the blood, methemoglobin levels of 89.6% and 96.5% were obtained at 38 and 68 minutes, respectively. Washing the cells in physiological saline took 30 minutes, after which the cells were resuspended in the guinea pig's plasma. The methemoglobin level at this time was 80.4%. Three hours and 15 minutes later the methemoglobin level was zero (Figure 3).

Determination of Lethal Doses of $NaNO_2$ for Guinea Pigs

Males. No deaths occurred when $NaNO_2$ was given at the rate of 60 mg./kg. to 10 guinea pigs. Deaths did occur, however, when $NaNO_2$ was given at the rate of 70 mg./kg. or more. In guinea pigs given $NaNO_2$, 100 mg./kg., the mortality was 100% (Table 3). The estimated LD_{50} was 79 mg./kg.

Signs of intoxication observed were hyperpnea, drowsiness, sprawling of hind legs while walking, cyanosis, lowering of body temperature, unsteady gait, unconsciousness, and gasping. These signs were followed by death or recovery. Although the majority of deaths occurred approximately an hour after administration of the $NaNO_2$, the range was from 36 to 70 minutes. One guinea pig gradually recovered after being comatose for an hour.

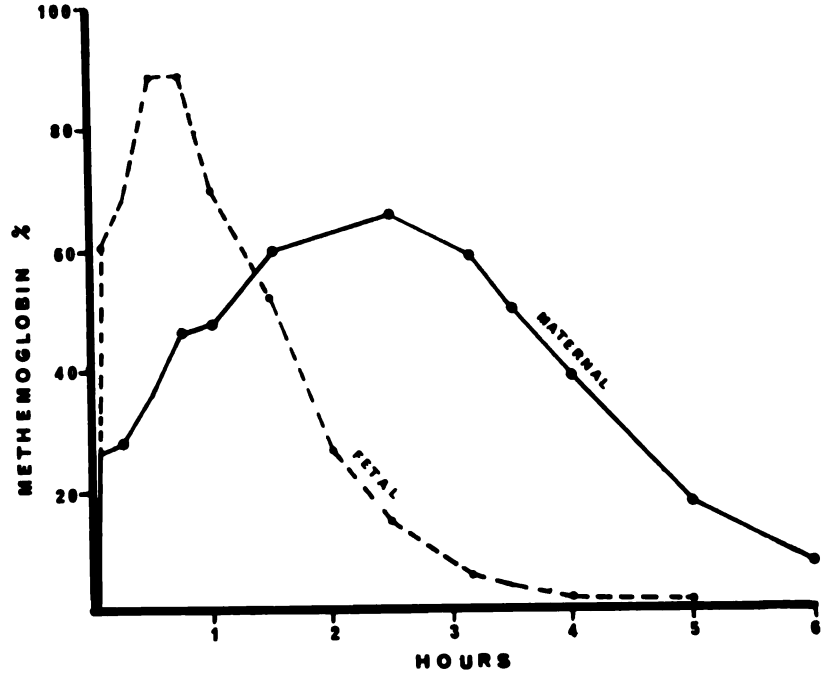

Figure 2. Methemoglobinization of guinea pig blood induced by $NaNO_2$, 40 mg./100 ml.

Table 2. In vitro methemoglobinization of blood from guinea pigs of different ages. Dose level of $NaNO_2$ equivalent to 40 mg./100 ml. of blood

	Methemoglobin Level (%) at Intervals													
Age	0 min.	5 min.	10 min.	15 min.	30 min.	45 min.	1 hr.	1.5 hrs.	2 hrs.	2.5 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.
1 day	0.0	44.6	---	53.4	45.3	50.0	53.3	34.5	10.5	5.0	0.0	0.0	0.0	---
3 days	0.2	23.4	57.6	52.3	63.7	---	70.9	---	61.6	---	---	4.8	6.4	4.7
11 days	1.3	---	47.0	60.6	74.2	---	76.5	81.5	72.0	58.3	43.3	10.6	4.1	0.0
3 weeks	0.3	1.3	23.9	39.6	25.9	36.9	54.0	81.7	79.3	---	50.5	1.0	3.6	0.6
9 weeks	0.0	0.3	56.3	55.0	63.2	77.0	75.7	77.9	67.9	---	42.9	12.9	4.0	1.3
4.5 months	0.0	---	20.0	23.2	36.0	---	49.0	47.2	66.4	---	63.1	42.2	7.2	0.0

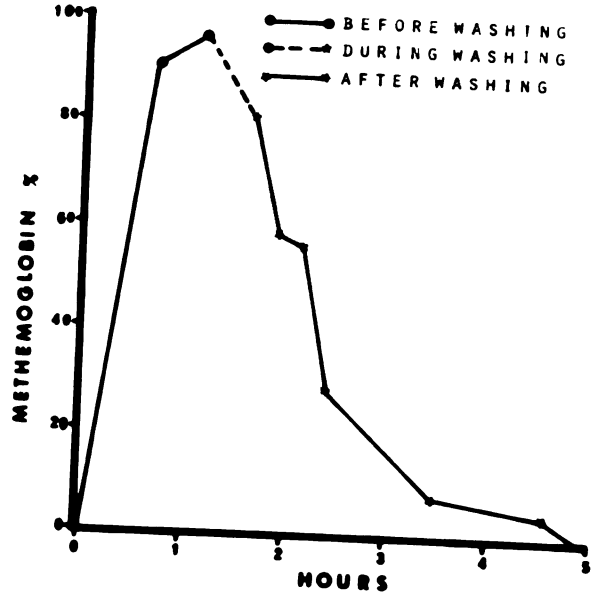

Figure 3. Methemoglobinization of guinea pig blood induced by $NaNO_2$, 80 mg./100 ml.

Table 3. Mortality rate in male guinea pigs produced by subcutaneous administration of $NaNO_2$

Dose (mg./kg.)	No. Injected	Mortality Rate (%)
100	10	100
90	10	90
80	10	60
70	10	10
60	10	0

Pregnant Females. The mortality rate was 100% when 70 mg./kg. of $NaNO_2$ was given to 4 pregnant guinea pigs; however, the survival rate was 100% when 60 mg./kg. of $NaNO_2$ was given to 36 pregnant females.

Fetuses. The data are given (Table 4). Fetuses died which were given $NaNO_2$ at levels of 73 mg./kg. or higher.

Determination of Levels of Methemoglobin and Plasma Nitrite after Administration of $NaNO_2$ to Guinea Pigs

Different Doses of $NaNO_2$ to Males. The degree of methemoglobinemia produced by different doses of $NaNO_2$ is given (Table 5). With a dose of $NaNO_2$ of 60 mg./kg., the highest level of methemoglobin was observed about an hour after subcutaneous administration. The methemoglobin was then gradually reduced to hemoglobin with only 2% methemoglobin present at 5 hours and 15 minutes after injection (Figure 4).

The plasma nitrite level reached a peak 7.5 to 15 minutes after subcutaneous administration of the $NaNO_2$ (Table 6).

Evaluation of Protective Effect of Methylene Blue on $NaNO_2$ Toxicosis. Methylene blue, 40 mg./kg., alone produced a small amount (2.3%) of methemoglobin. The methemoglobin level was only 3.8% when 60 mg./kg. of $NaNO_2$ and 40 mg./kg. of methylene blue were given simultaneously (Table 7).

In the guinea pig given $NaNO_2$ at the rate of 100 mg./kg. and methylene blue, 10 mg./kg., the methemoglobin level in the blood was 7.5% at 75 minutes after treatment. There was no methemoglobin in the blood sample taken before administration of the $NaNO_2$.

Table 4. Determination of lethal dose of $NaNO_2$ for fetuses of guinea pigs

Dam No.	Length of Gestation (days)	Fetus No.	$NaNO_2$ (mg./kg.) subcu.	Body Wt. (Gm.)	Remarks
1	55	1-1	23	88	Live
		1-2	61	82	Live
2	55	2-1	0	70	Live
		2-2	73	82	Dead
		2-3	109	73	Dead
		2-4	141	71	Dead
3	60	3-1	92	54	Dead
		3-2	71	57	Live
		3-3	0	59	Live
		3-4	0	53	Live

Table 5. Methemoglobinemia induced by $NaNO_2$ in guinea pigs*

Dose (mg./kg.) subcu.	MetHb. Levels (%) at Intervals			
	30 min.	60 min.	90 min.	120 min.
10	2	3	1	1
20	12	8	5	3
30	32	25	16	4
40	49	58	46	32
60	40	67	66	63
70	55	75	72	31
80	70	84**	---	---

* Results given for 1 representative animal for each dose.

** Died after blood sample was drawn.

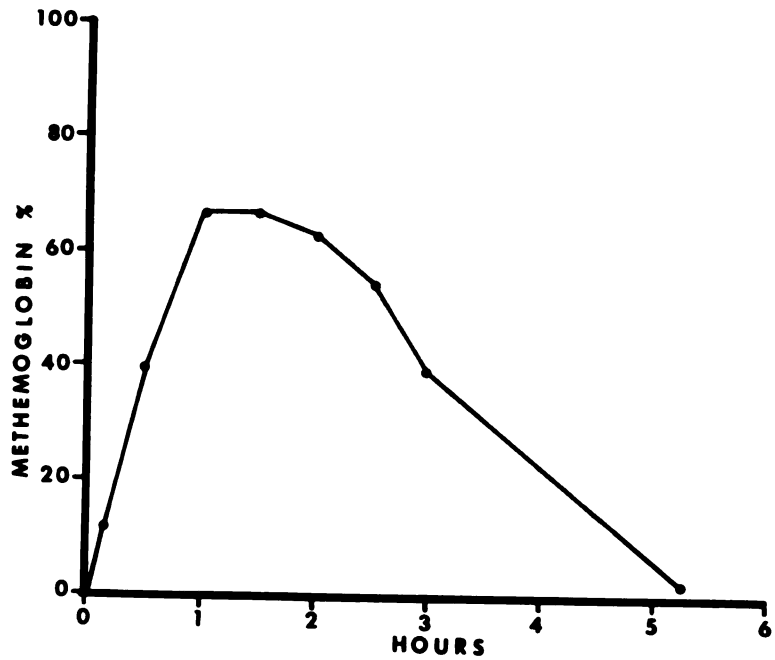

Figure 4. Methemoglobinemia in guinea pig induced by $NaNO_2$, 60 mg./kg. (subcutaneously).

Table 6. Plasma nitrite nitrogen levels after subcutaneous administration of $NaNO_2$ to guinea pigs

Guinea Pig No.	Dose (mg./kg.)	Plasma Nitrite Nitrogen (mg./100 ml.) at Intervals (min.)							
		0	7.5	15	22.5	30	60	90	120
1	40	0	---	1.35	---	0.90	---	---	---
2	60	0	---	---	---	1.15	0.6	0.6	0.0
3	60	0	---	2.30	---	1.50	---	---	---
4	60	0	2.15	1.65	1.65	---	---	---	---
5	80	0	---	2.70	---	2.45	---	---	---

Table 7. Methemoglobinemia in male guinea pigs produced by $NaNO_2$ and methylene blue

Guinea Pig No.	Treatment	Methemoglobin Levels (%) at Intervals (Hrs.) 0	1	2	3
1	$NaNO_2$, 60 mg./kg.	0	66.7	62.7	39.2
2	Methylene blue, 40 mg./kg.	0	0.9	2.3	0.0
3	$NaNO_2$, 60 mg./kg., and methylene blue, 40 mg./kg.	0	3.8	2.3	0.8

Determination of Methemoglobin and Plasma Nitrite in Maternal and Fetal Blood. The degree of methemoglobinemia produced by $NaNO_2$ (40 or 60 mg./kg.) in pregnant guinea pigs is shown (Table 8). The methemoglobin and plasma nitrite values after administration of $NaNO_2$ (40 mg./kg.) in pregnant guinea pigs are given (Table 9). The fetal blood samples contained small amounts of methemoglobin and plasma nitrite. The guinea pigs given $NaNO_2$ at the rate of 60 mg./kg. had higher levels of methemoglobin than those receiving 40 mg./kg. of $NaNO_2$ (Table 10). In anesthetized dams in which repeated samples were taken, the highest methemoglobin level in maternal blood was 51.5%, while the highest in fetal blood was 10.6% (Figure 5). All the fetuses were alive.

Pathologic and Embryotoxic Effects of $NaNO_2$ Toxicosis

Experiment 1. The body temperature of the guinea pigs was lowered approximately 1 C. by 1 hour after administration of the $NaNO_2$ (50 mg./kg.); the body temperature returned to normal within the next 3 hours (Table 11). All guinea pigs, those given $NaNO_2$ as well as the controls, gave birth to normal litters.

Experiment 2. Methemoglobin or plasma nitrite was not detected in the blood before, 12 or 24 hours after administration of the chemicals, either in the experimental or control animals. All the fetuses were alive.

No visible gross lesions were detected during postmortem examination. On microscopic examination, centrolobular hepatic lipidosis was observed in the experimental animal killed 24 hours after treatment. The remaining organs appeared normal.

Table 8. Methemoglobinemia in pregnant guinea pigs produced by $NaNO_2$*

Dose of $NaNO_2$ (subcu.)	MetHb. (%) at Intervals				
	0 min.	10 min.	20 min.	40 min.	1 hr.
40 mg./kg.	0	6.5	30.4	38.9	11.8
60 mg./kg.	0	2.0	30.1	60.0	68.8

* One representative guinea pig in late pregnancy was selected for each dose.

Table 9. Methemoglobin and plasma nitrite levels in the maternal and fetal blood after administration of $NaNO_2$ (40 mg./kg.) to dam*

Time of Samples after Administration of $NaNO_2$ (min.)	Maternal Blood		Fetal Blood	
	MetHb. (%)	Plasma Nitrite Nitrogen (mg./100 ml.)	MetHb. (%)	Plasma Nitrite Nitrogen (mg./100 ml.)
0	0.0	0.00	0.0	0.00
20	4.0	0.18	0.0	0.04
40	8.0	0.13	2.6	0.06
60	38.5	0.10	0.3	0.04
80	29.2	0.03	1.1	0.02

* Dam was anesthetized and hysterotomy was performed in order to obtain fetal blood.

Table 10. Methemoglobin and plasma nitrite levels in the maternal and fetal blood after administration of $NaNO_2$ (60 mg./kg.) to dam

Guinea Pig No.	Time of Samples after Administration of $NaNO_2$ (min.)	Maternal Blood		Fetal Blood	
		MetHb. (%)	Plasma Nitrite Nitrogen (mg./100 ml.)	MetHb. (%)	Plasma Nitrite Nitrogen (mg./100 ml.)
1	0	0.0	0.00	0.0	0.00
2	20	47.4	0.15	7.3	---
3	60*	64.0	0.08	20.8	0.07
4	100*	17.4	0.08	---	---
5	140*	9.0	0.00	4.0	0.00

* Fetuses were dead.

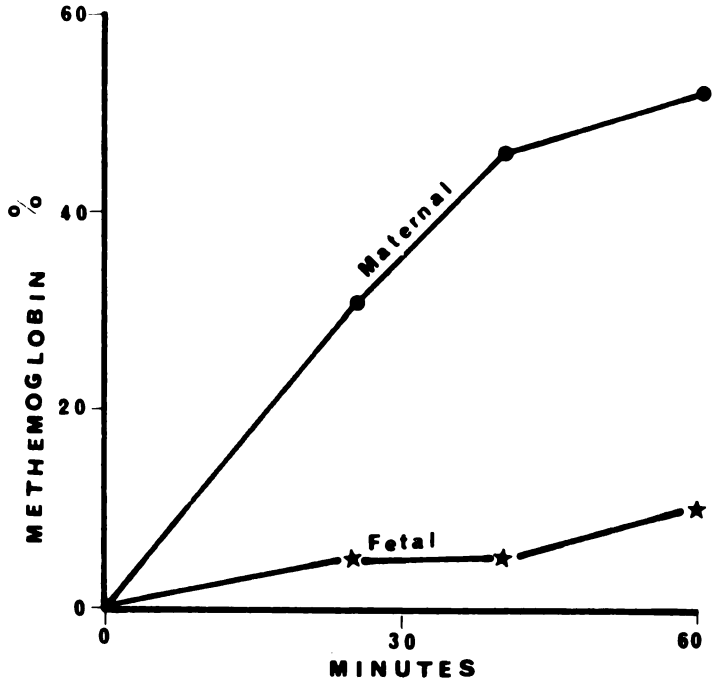

Figure 5. Methemoglobinemia in the pregnant guinea pig induced by $NaNO_2$, 60 mg./kg. (subcutaneously).

Table 11. Effect on body temperature of subcutaneous administration of $NaNO_2$ (50 mg./kg.) to pregnant guinea pigs

Guinea Pig No.	$NaNO_2$ (mg./kg.)	NaCl (mg./kg.)	Body Temperature (C.) Before Treatment	After Treatment at Intervals (hrs.) 0.5	1	2	4
1	---	60	39.7	39.5	39.5	39.8	40.1
2	---	60	38.8	38.5	38.7	38.9	39.0
3	60	---	38.9	38.3	37.8	37.8	38.5
4	60	---	38.9	38.7	38.2	38.4	38.9
5	60	---	39.1	37.8	37.4	37.3	38.2
6	60	---	39.7	38.0	37.3	38.7	39.0

Experiment 3. The reproductive performance is tabulated (Table 12).

Experiment 4. Neither methemoglobin nor plasma nitrite was found in any of the blood samples from the control animals.

In the experimental animals methemoglobin was present in both maternal and fetal blood up to 6 hours after administration of $NaNO_2$. After this no significant amount of methemoglobin could be detected (Table 13). However, no plasma nitrite was found in either the maternal or fetal blood samples taken at 3 hours after administration of $NaNO_2$. There was a slight absolute leukocytosis in the guinea pigs given $NaNO_2$; no significant changes appeared in the leukocyte counts of the guinea pigs given NaCl.

No Heinz bodies were demonstrated in the blood stained with new methylene blue either in the experimental or control animals.

All the fetuses were alive in both the experimental and control animals at 0.25 and 1.5 hours after treatment. Fetuses of the treated guinea pigs, examined at 3 hours and onward following administration of $NaNO_2$, had a mortality rate of 72%. No fetal deaths occurred in the control animals (Table 14).

Gross Lesions. The blood, mucous membranes and viscera had a dark brown appearance in the experimental animals killed at 1.25, 1.5 and 3 hours after administration of $NaNO_2$. Placentas of the experimental animals killed 18 hours after treatment and onward were pale in contrast to the bright red placentas of the control animals. Placentas from experimental animals killed at 24 hours and onward were friable.

Fetuses of the nitrite-treated animals killed at 24, 24.5, 48 and 56 hours after treatment were dead and edematous (Figures 6 and 7).

Table 12. Reproductive performance of guinea pigs given $NaNO_2$ or NaCl subcutaneously

Treatment (60 mg./kg.)	Time of Abortion or Parturition	Condition of Fetuses			Remarks
		Live	Dead	Total	
$NaNO_2$	< 24 hrs.	1	3	4	Premature
$NaNO_2$	< 24 hrs.	0	2	2	Premature
$NaNO_2$	4 days	0	5	5	Premature
$NaNO_2$	---	-	-	-	Not pregnant
NaCl	6 days	3	0	3	Normal birth
NaCl	---	-	-	-	Died*

* Three days after treatment, she died due to pneumonia.

Table 13. Methemoglobin and plasma nitrite levels in the maternal and fetal blood after administration of $NaNO_2$ (60 mg./kg.) to dam

Time of Sampling after Administration of $NaNO_2$ (hrs.)	Maternal Blood		Fetal Blood	
	MetHb.(%)	Plasma NO_2-N (mg./100 ml.)	MetHb.(%)	Plasma NO_2-N (mg./100 ml.)
0.25	27.0	0.19	1.1	0.02
1.50	23.3	0.07	9.2	0.02
3.00	18.6	0.00	8.8	0.00
6.00	0.2	0.00	2.3	0.00
12.00	0.0	0.00	0.0	0.00
18.00	0.0	0.00	0.0	0.00
24.00	0.0	0.00	0.0	0.00
24.50	0.0	0.00	0.0	0.00
48.00	0.0	0.00	0.0	0.00
56.00	0.0	0.00	0.0	0.00

Table 14. Fetal mortality after subcutaneous administration of $NaNO_2$ or NaCl (60 mg./kg.) to pregnant guinea pigs

Guinea Pig No.	Time of Examination after Treatment (hrs.)	Fetuses ($NaNO_2$)			Fetuses (NaCl)		
		Live	Dead	Average Wt. (Gm.)	Live	Dead	Average Wt. (Gm.)
1	0.25	3	0	86	7	0	52
2	1.50	7	0	35	2	0	105
3	3.00	0	4	99	2	0	94
4	6.00	0	3	108	4	0	68
5	12.00	6	0	6	4	0	10
6	18.00	0	5	74	4	0	87
7	24.00	1	1	35	5	0	86
8	24.50	0	4	82	5	0	99
9	48.00	0	3	35	3	0	22
10	56.00	0	5	76	3	0	75

Figure 6. Fetus of $NaNO_2$-treated (60 mg./kg.) guinea pig killed 24.5 hours after treatment. Notice subcutaneous edema and discoloration (arrow).

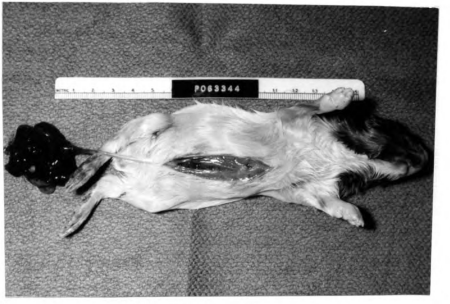

Figure 7. Fetus of control guinea pig killed same time as in Figure 6.

Microscopic Lesions

Liver. In experimental animals a mild degree of hepatic lipidosis was confirmed by Oil Red O stain. Lipidosis was more prominent in the centrolobular areas (Figures 8 and 9).

Placenta. Placentas of experimental animals killed at 0.25, 1.5 and 3 hours were hyperemic. Coagulative necrosis was observed in the placentas of experimental animals killed at 18 hours and onward (Figures 10 and 11). The extent of the lesions progressed with time.

Prevention of Acute Toxicity of $NaNO_2$ in Pregnant Guinea Pigs by Treatment with Methylene Blue

Group 1. The fetuses were apparently protected by the administration of the methylene blue to the dams at the time of nitrite administration (Table 15).

Group 2. The guinea pigs given $NaNO_2$ aborted 3 to 4 days after treatment, while those given $NaNO_2$ and methylene blue or methylene blue alone farrowed normally (Table 16).

Effects of $NaNO_2$ Administration on Pregnant Guinea Pigs During First 30 Days of Gestation

Group 1. Among the nitrite-treated guinea pigs only 2 fetuses died. Results for this group are given (Table 17).

Group 2. The guinea pig killed 8 days after $NaNO_2$ administration had 3 dead fetuses. The fetuses were all alive in the experimental as well as the control animals killed on the 2nd, 3rd and 11th days. The

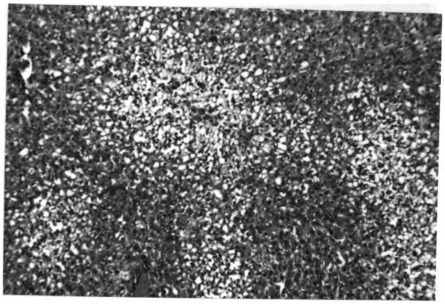

Figure 8. Hepatic lipidosis in $NaNO_2$-treated guinea pig. H & E stain. x 75.

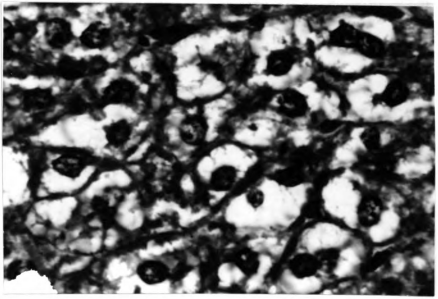

Figure 9. Higher magnification of a portion of Figure 8. H & E stain. x 750.

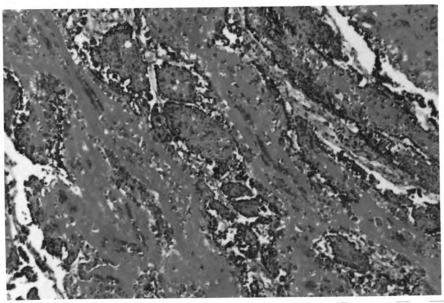

Figure 10. Necrosis of the placenta in $NaNO_2$-treated guinea pig. H & E stain. x 75.

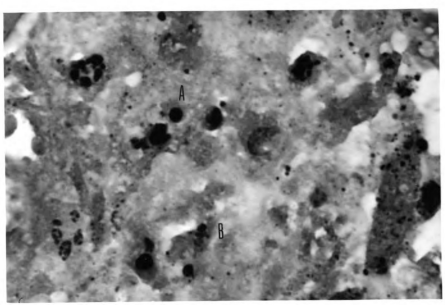

Figure 11. Higher magnification of a portion of Figure 10, to show karyorrhexis (A) and pyknosis (B). H & E stain. x 750.

Table 15. Effect of $NaNO_2$ or NaCl (60 mg./kg.) on the fetuses of guinea pigs treated with methylene blue

Stage of Gestation (days)	Methylene Blue (mg./kg.)	$NaNO_2$ or NaCl	Time of Examination after Treatment (hrs.)	Fetuses			
				Live	Dead	Total	Total Weight (Gm.)
62	10	$NaNO_2$	3	5	1	6	550
50	10	NaCl	3	5	1	6	280
66	10	$NaNO_2$	24	4	0	4	390
65	10	NaCl	24*	3	0	3	250
55	10	$NaNO_2$	100	4	0	4	220
55	10	NaCl	100	5	0	5	225

* Farrowed normally within 24 hours after treatment.

Table 16. Reproductive performance of pregnant guinea pigs treated with $NaNO_2$ and methylene blue

Methylene blue mg./kg. (I.P.)	$NaNO_2$ mg./kg. (subcu.)	Time* of Abortion or Parturition	Condition of Fetuses	
			Live	Dead
10	60	9 days	4	0
10	60	9 days	4	0
10	60	4 days	3	0
---	60	3 days	0	3
---	60	4 days	0	2
10	---	15 days	3	1

* Post-treatment.

Table 17. Effect of $NaNO_2$ administered subcutaneously to guinea pigs on the 10th day of pregnancy (estimated)

Guinea Pig No.	$NaNO_2$ (mg./kg.)	NaCl (mg./kg.)	Exam. of Fetuses after Treatment	Condition of Fetuses	
				Live	Dead
1	60	---	7 days	6	0
2	60	---	14 days	4	2
3	60	---	at term	4	0
4	---	60	at term	3	0
5	---	60	at term	4	0

2 experimental and 1 control animals which were kept under observation farrowed without any apparent complications (Table 18).

Group 3. The fetuses were alive in the nitrite-injected animals killed on the 2nd, 7th and 14th days, as well as the control animal killed on the 7th day of the experiment. Two experimental and 1 control animal, which were not killed, farrowed normally. One of the control animals was not pregnant (Table 19).

Guinea Pigs 5 and 7, which were given $NaNO_2$, each had 1 young with deformity of the hind leg (Figure 12).

Maternal and Fetal Blood P_{O_2}, P_{CO_2}, pH and Methemoglobin Values after Administration of $NaNO_2$

In guinea pigs given $NaNO_2$, 60 mg./kg., there was a corresponding reduction in the maternal and fetal blood P_{O_2} values (Figure 13) and an elevation in P_{CO_2} values (Figure 14) with the increases of methemoglobin levels in the maternal blood (Figure 15).

The reduction of P_{O_2} values in the maternal and fetal blood of guinea pigs given $NaNO_2$, 45 mg./kg., was less than those given $NaNO_2$, 60 mg./kg.

There was a drop in the fetal blood pH after administration of $NaNO_2$, 60 mg./kg., to the dam (Table 20).

Table 18. Effect of $NaNO_2$ administered subcutaneously to guinea pigs on the 20th day of pregnancy (estimated)

Guinea Pig No.	$NaNO_2$ (mg./kg.)	NaCl (mg./kg.)	Exam. of Fetuses after Treatment	Condition of Fetuses	
				Live	Dead
1	60	---	2 days	3	0
2	---	60	2 days	5	0
3	60	---	3 days	2	0
4	---	60	8 days	5	0
5	60	---	8 days	0	3
6	60	---	11 days	5	0
7	---	60	at term	5	0
8	60	---	at term	4	0
9	60	---	at term	3	0

Table 19. Effect of $NaNO_2$ administered subcutaneously to guinea pigs on the 30th day of pregnancy (estimated)

Guinea Pig No.	$NaNO_2$ (mg./kg.)	NaCl (mg./kg.)	Exam. of Fetuses after Treatment	Condition of Fetuses	
				Live	Dead
1	60	---	2 days	4	0
2	60	---	7 days	5	0
3	---	60	7 days	4	0
4	60	---	14 days	2	0
5	60	---	at term	2*	1
6	---	60	at term	3	0
7	60	---	at term	3*	0
8	---	60	at term	2	0

* One young in the litter had a deformity of the hind leg.

Figure 12. Young (1 day old) of the guinea pig (No. 7) given $NaNO_2$, 60 mg./kg. (subcutaneously). Notice deformity of hind leg (arrows).

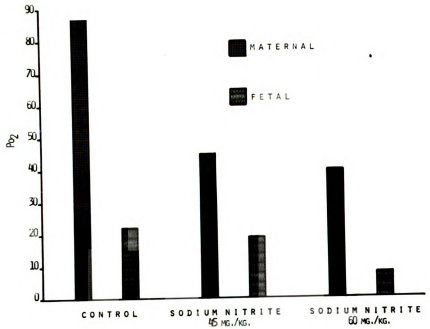

Figure 13. The mean P_{O_2} value of treated and untreated guinea pigs.

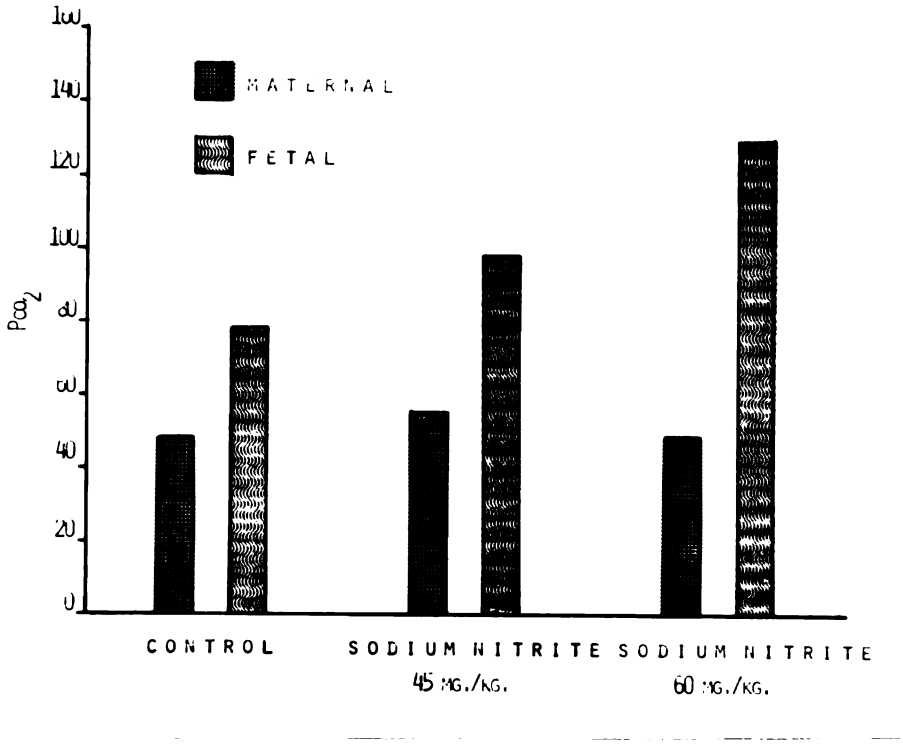

Figure 14. The mean P_{CO_2} value of treated and untreated guinea pigs.

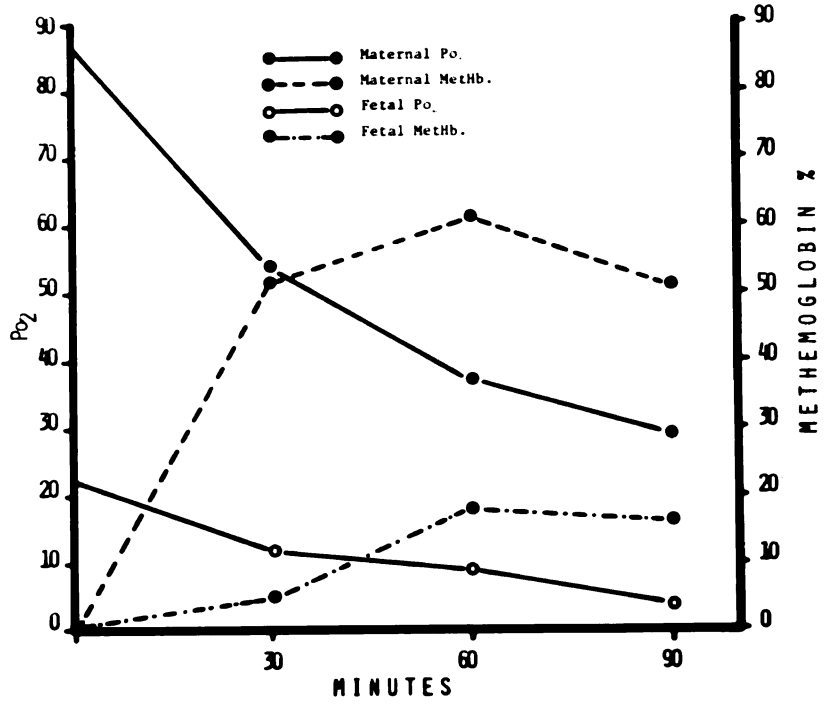

Figure 15. The effect of $NaNO_2$ (60 mg./kg., subcutaneously) on maternal and fetal methemoglobin and P_{O_2} values.

Table 20. Maternal and fetal blood pH of $NaNO_2$-treated (subcu.) and untreated guinea pigs

Guinea Pig No.	$NaNO_2$ (mg./kg.)	Sampling Time After Admin. of $NaNO_2$ (min.)	Blood pH	
			Maternal	Fetal
1	0	---	7.44	7.18
2	0	---	7.19	7.25
3	0	---	7.44	7.20
4	45	30	7.22	7.03
5	45	60	7.42	7.01
6	45	90	7.37	7.27
7	60	30	7.42	6.88
8	60	60	7.26	6.80
9	60	90	7.37	6.93

DISCUSSION

Since the formation of methemoglobin is the major factor in the pathogenesis of nitrite toxicosis, it was of prime importance to first study the methemoglobinization of blood *in vitro*. The data obtained were then used to build a model for the *in vivo* studies.

As reported, 0.5 to 0.7 mole of $NaNO_2$ converts 1 mole of oxyhemoglobin to methemoglobin (Austin and Drabkin, 1935). The red blood cell absorbs the nitrite ion which causes the oxidation of hemoglobin to methemoglobin, consequently lowering the nitrite content of the plasma. The reducing enzymes present in the normal RBC's simultaneously reduce the methemoglobin formed back to hemoglobin. However, the oxidation of hemoglobin continues as long as the nitrite ions are in contact with the RBC's. Thus, higher doses of $NaNO_2$ increased not only the level of methemoglobin but also the period of reduction of methemoglobin to hemoglobin.

There was no significant variation in the rate of methemoglobin formation in the 3 different concentrations of red blood cells (Figure 1). However, it was apparent that the reduction of methemoglobin to hemoglobin in blood diluted 50% with plasma was slower than in the whole blood. This may be explained by the fact that there was twice as much nitrite per red blood cell in the diluted blood sample as in the whole blood. The information gained here indicates that in an anemic animal, a dose of nitrite could produce a higher degree of methemoglobinemia than in a healthy animal.

The enzyme, methemoglobin reductase, utilizes reduced DPN and TPN which are derived from glycolysis and the phosphogluconate oxidative pathway (White, Handler and Smith, 1959). Thus, the absence of reduction of methemoglobin in the sample of RBC's suspended in physiological saline solution was probably due to the exhaustion of DPNH and TPHN, which suggests that plasma plays a role in the reduction of methemoglobin to hemoglobin.

Lower amounts of methemoglobin reductase in fetal blood (Ross, 1963) is not adequate to explain the faster and higher level of methemoglobin formation and quicker reduction in comparison to maternal blood as observed in vitro by the author. If the difference were simply due to a decreased amount of the reductase, there would have been a slower reduction of methemoglobin rather than faster, as was observed and shown in Figure 2.

It has been reported that when 80 to 90% of the hemoglobin is converted to methemoglobin, death occurs as a result of anoxia (Bodansky, 1951). However, it was not clear whether the anoxia was preceded by destruction of the intraerythrocytic enzymic system. In order to clarify this, a high level (96%) of methemoglobin was produced in vitro, after which the cells were thoroughly washed so that extraerythrocytic nitrite would not be in contact with the RBC's. The reduction of methemoglobin to hemoglobin did not take any longer in the blood containing a high level of methemoglobin than it did in the sample with only a moderate amount (Table 1). This observation supports the view that even in the case of a high degree of methemoglobinemia, the enzymic system remains intact.

Following subcutaneous administration, $NaNO_2$ was absorbed so rapidly that the plasma nitrite level reached a peak between 7.5 and 15 minutes. As the nitrite ions came in contact with the erythrocytes, the nitrite content of the plasma was gradually reduced to zero. The highest level of methemoglobin was observed about an hour after treatment, which corresponded with the results of the *in vitro* studies.

Plasma nitrite was detected in the fetuses. This proves that the nitrite ion can pass through the guinea pig placenta. Maternal plasma nitrite values, however, were higher than fetal plasma nitrite values, thus indicating that there may be a partial placental barrier to the transport of nitrite to the fetuses. It is possible that some of the nitrite ions form a complex with the plasma protein, which cannot pass through the placenta.

Pregnant guinea pigs given $NaNO_2$, 50 mg./kg., had a normal parturition, and there was no detectable effect on the fetuses. When pregnant guinea pigs were given $NaNO_2$, 60 mg./kg., abortions with fetal mortality occurred 1 to 4 days later. It is important to emphasize that there was a sharp demarcation between the doses of $NaNO_2$ which produced abortion and those which had no effect upon reproduction.

As a general rule, the fetuses died between 1.5 and 3.5 hours after the dam was given $NaNO_2$, 60 mg./kg., subcutaneously. By correlating the information gained from Figures 4 and 5, it is apparent that the fetal deaths occurred when maternal and fetal methemoglobin levels were at their peak, but not during the plasma nitrite peak which was attained between 7.5 and 15 minutes after administration of the $NaNO_2$ (Table 6). This indicates that nitrite as such was not lethal to the fetuses, but rather that its ability to form methemoglobin, with the resulting hypoxia

or anoxia, was responsible for the fetal deaths. At the time of the fetal deaths there were no significant changes in the placentas. Upon death of the fetuses, the fetal blood circulation was stopped, thus interrupting the normal mechanism of blood flow in the placenta. It is believed that the pathologic changes seen in the placentas, therefore, developed after the death of the fetuses. If this be true, the necrotic changes observed in the placentas were a consequence, rather than a cause, of the fetal deaths.

Methylene blue seems to be a very good protective agent against nitrite poisoning, since only a small amount of methemoglobin was detected in nitrite-treated animals receiving it. In the body, methylene blue is reduced to leuco-methylene blue and is readily available for the reduction of methemoglobin to hemoglobin (Jones, 1965), the effect being the reduction of the methemoglobin level in the blood. In pregnant guinea pigs given $NaNO_2$ (60 mg./kg.) and treated with methylene blue (10 mg./kg.) no fetal deaths occurred.

The findings strongly suggest that fetal deaths were produced due to methemoglobinemia which led to hypoxia. In order to confirm that fetuses were dying because of the hypoxia, the maternal and fetal P_{O_2} and P_{CO_2} of control and nitrite-treated animals were determined. The data obtained definitely indicated that there was a lower P_{O_2} along with a higher P_{CO_2} value in the fetuses of guinea pigs treated with $NaNO_2$ (60 mg./kg.) in comparison to the control animals. Fetal P_{O_2} values from dams treated with $NaNO_2$ at the rate of 45 mg./kg. were not appreciably lowered. It seems that there is a critical level of maternal methemoglobin up to which fetal P_{O_2} is not affected, but beyond this point fetal P_{O_2} is reduced and the end result is a hypoxic condition in fetuses.

Severe degrees of hypoxia obviously may lead to death.

In this study when $NaNO_2$, 60 mg./kg., was given to guinea pigs on the 30th day of pregnancy, a deformity of the hind leg was observed among 13% of the young. The author's finding is similar to that of Grabowski (1961a), in which teratogenesis was observed when chick embryos were exposed to hypoxia.

The administration of $NaNO_2$, 60 mg./kg., to guinea pigs in the first half of pregnancy produced less fetal mortality than when given in the last half of pregnancy. This could be explained by the fact that in the latter stages of pregnancy the fetuses comprise a greater percentage of the body weight of the pregnant guinea pig than earlier in pregnancy. Thus, even though the same dose of $NaNO_2$ was given on a body weight basis (mg./kg.) there would be less nitrite per red blood cell in early pregnancy than in late pregnancy. However, there may be some other factors responsible for the difference; but further investigation is needed in order to clarify this.

In the guinea pig, sodium nitrite, depending upon the dose, produced a mild to severe degree of methemoglobinemia but did not produce Heinz bodies. This observation is comparable to work done in pigs (Sinha and Sleight, 1968).

SUMMARY AND CONCLUSIONS

This work was designed to study the effects of acute nitrite toxicosis on reproduction in guinea pigs. Basic to the characterization of these effects was a series of experiments on the extent of methemoglobin formation caused by $NaNO_2$ *in vitro* and *in vivo*. The pathologic and embryotoxic effects of acute $NaNO_2$ toxicosis at various stages of gestation were studied. Maternal and fetal P_{O_2} and P_{CO_2} values were determined after administration of $NaNO_2$ to the dam. From the experimental results, the following observations and conclusions can be made:

1. When pregnant guinea pigs were given $NaNO_2$, 60 mg./kg., abortion with fetal mortality occurred.

2. The fetal deaths occurred when maternal and fetal methemoglobin levels were at their peak but not during the plasma nitrite peak. Methemoglobinemia led to hypoxia which caused the fetal deaths.

3. Following subcutaneous administration of $NaNO_2$, 60 mg./kg., the plasma nitrite level reached a peak between 7.5 and 15 minutes. The highest level of methemoglobin was observed about an hour after treatment.

4. There were lower P_{O_2} and higher P_{CO_2} values in the fetuses of the guinea pigs treated with $NaNO_2$, 60 mg./kg., than in fetuses of the control animals.

5. The pathologic changes seen in the placentas were a consequence, rather than a cause, of the fetal deaths.

6. In the pregnant guinea pigs given $NaNO_2$, 60 mg./kg., and treated with methylene blue, 10 mg./kg., no fetal deaths occurred.

7. When $NaNO_2$, 60 mg./kg., was given to guinea pigs on the 30th day of pregnancy, a deformity of the hind leg was observed among 13% of the young.

8. When dams were given $NaNO_2$ subcutaneously, maternal plasma nitrite values were higher than fetal plasma nitrite values, which indicates that there may be a partial placental barrier to transport of nitrite to the fetuses.

9. Even with a high degree (96%) of methemoglobinization, the enzyme system was capable of reducing methemoglobin to hemoglobin.

10. Studies on methemoglobinization of diluted blood indicated that methemoglobinemia is potentially more harmful in anemic animals.

11. In the guinea pig, $NaNO_2$ did not produce Heinz bodies.

12. The administration of $NaNO_2$, 60 mg./kg., to guinea pigs in the first half of pregnancy produced less fetal mortality than when given in the last quarter of pregnancy. In order to pinpoint the factors responsible for the difference, a further investigation is needed.

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