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# Investigations on teratogenic effects of dietary nitrite in the rat and excess vitamin A in the dog

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# INVESTIGATIONS ON TERATOGENIC EFFECTS OF DIETARY NITRITE IN THE RAT AND EXCESS VITAMIN A IN THE DOG

by

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# INTRODUCTION

A rapid expansion in experimental teratogenesis resulted from the thalidomide disaster. Thalidomide had been considered as almost innocuous in man previously and yet produced maimed infant victims as a result of exposure to the drug by pregnant females. The unusual characteristic deformities produced by thalidomide were instrumental in defining thalidomide as the responsible agent.

Many drugs have been used in the experimental production of congenital anomalies, but many naturally occurring congenital anomalies have not been defined on an etiologic basis. Multiple, rather than single, external and genetic factors may be important in determining naturally occurring malformations.

Congenital malformations can be induced in mammals by maternal dietary deficiencies. Deficiency of the mother's diet in vitamin A, riboflavin, folic acid, pantothenic acid, vitamin $B_{12}$, vitamin D and vitamin E have been used experimentally to induce congenital malformations in the young. Congenital malformations can be obtained by dietary deficiencies only if the mother is in a borderline deficiency state. It can be easily demonstrated that deprivation of a specific essential nutrient results in sterility, resorption of embryos or the birth of dead or nonviable young. It is equally easy to show improvement in reproductive performance by utilization

of adequate diets. However, it is difficult to create a borderline deficiency that damages the embryo without killing it. These borderline deficiencies have been created experimentally in the laboratory.

Domesticated animals may be kept in marginal deficiency states or may be exposed to other factors that may inhibit utilization of, or cause a depletion of otherwise normal quantities of essential factors. Vitamin A stores and utilization have been reported to be affected by nitrate and nitrite, but results are equivocal and vary with the species. Vitamin A deficiency has been shown to have a teratogenic effect. The effect of nitrite in pregnant rats, some of which will have marginal vitamin A deficiency, will be investigated in this study.

Nitrite oxidizes the ferrous iron of hemoglobin with the production of methemoglobin which does not transport oxygen. Anoxic conditions can be produced depending upon the amount of hemoglobin that is converted to methemoglobin. Anoxia has been reported as an experimental teratogenic agent.

Certain ions have an inhibitory effect on the uptake of iodides by the thyroid with the result that hypothyroidism may be produced. Nitrate and possibly nitrite have been shown to have this effect. Hypothyroidism has been reported to have a teratogenic effect.

The use of vitamin A in increasingly larger doses as a therapeutic agent has become a common veterinary practice.

Excess vitamin A has a proven teratogenic effect in rodents. There have been few reports on the use of domestic animals in experimental teratology. The existence of a teratogenic effect of excess vitamin A in the canine will be investigated in this preliminary study.

# PART I. EFFECTS OF DIETARY NITRITE IN THE PREGNANT RAT

# REVIEW OF LITERATURE

## Historical

The review of literature in this portion covers the historical development of experimental teratology and concerns either initial or major contributions to mammalian teratology with reference, on occasion, made to reports involving the human.

Teratology (Hoerr and Osel, 1956) is the science of malformations and monstrosities. Warkany (1961) states that the term "congenital malformations" should be applied to gross, structual anomalies present at birth, irrespective of their etiology or morphogenesis.

Experimental production of congenital malformations is not new. As early as 1891 Dareste produced abnormalities in the chicken embryo. More recently the experimental production of malformations in the mammalian fetus has been reported. Hale (1933, 1935, 1937) reported the teratogenic effect of maternal vitamin A deficiency in pigs.

Teratogenic procedures carried out in laboratory animals date back to 1940. Giroud and de Rothschild (1951) produced cataracts in the offspring of rats by the administration of thyroxine orally to the female rats before and during pregnancy. Fraser and Fainstat (1951) produced cleft palate in mice as a result of cortisone injections. They observed differences in the strain response to teratogenic doses of

cortisone. Kalter (1954) and Fraser et al. (1957) pointed out the contributions made by the genetic and environmental backgrounds of the embryo when exposed to an environmental agent. They used various crosses between two strains of mice that showed very different responses in congenital cleft palate production due to cortisone. Results indicated that both genotype and uterine environment of the embryo were important in determining the probability that an embryo would get cleft palate. Courrier et al. (1951) showed increased resorptions in rats as a result of cortisone injections administered to pregnant females. Raynaud (1943) found that estradiol administered to female mice inhibited the development of eyelids in the fetuses. It has been shown that some human female fetuses, whose mothers received synthetic progestins in the treatment of threatened abortion, were born with masculinized genitalia (Wilkins et al., 1958). Curtis and Grant (1964) have also reported this masculinization effect in female canine fetuses. Chomette (1955) had shown that the rabbit was a suitable animal for teratogenic experiments employing insulin. Malformations consisted of microcephaly and ectopia cordis.

Teratogenic effects have been produced by physical agents. Ingalls et al. (1950, 1952) showed that reduced atmospheric pressure resulted in increased incidence of malformations in litters of pregnant mice. However, under certain experimental conditions anoxia has counteracted teratogenic agents. Russell

and Russell (1954) found that hypoxia markedly protected mice against irradiation-induced abnormalities. Smith (1957) produced abnormalities in the young of pregnant hamsters that were subjected to severe hypothermia. Trasler et al. (1956) reported on the production of cleft palate in mice by puncture of the amniotic sac on day 13 of gestation. These cleft palates appeared to have resulted from a loss of amniotic fluid, which constricted the embryo, pushing the head down on the chest and forcing the lower jaw upward. Thus the tongue was forced between the palatine shelves, which therefore could not fuse.

Goldstein and Murphy (1929), among others, reported on the effect that x-rays had on human embryos that were exposed in utero to therapeutic doses. Some of the children born after such procedures showed microcephaly, microphthalmus, spasticity or mental retardation and sometimes malformations in the genital and the skeletal systems.

Warkany and Schraffenberger (1947) produced a variety of congenital malformations in the offspring of female rats by exposing them to roentgen rays on certain days of pregnancy. Skeletal malformations were common.

Haskin (1948) administered a radiomimetic and cytotoxic nitrogen mustard to pregnant rats and produced various malformations in the fetuses.

Diamond et al. (1960) reported that the therapeutic use of busulfan, a polyfunctional alkylating agent, has induced congenital malformations in human embryos. Harelip and cleft palate, hydrocephalus, encephalocele, anencephaly and other defects have been observed in affected embryos or infants.

Metaphase inhibitors have shown teratogenic activity in animals. Ferm (1963) injected colchicine intravenously into the pregnant hamster on the eighth day of gestation and obtain-

ed high morbidity with the survivors having exencephaly and skeletal abnormalities. Eye defects were also observed with colchicine, vinblastine and vincristine (Ferm, 1964). Podophyllotoxin and podophyllin caused resorptions of the fetal rat (Thiersch, 1963). A clinical report by Cullis (1962) describes a woman who took "slimming tablets" containing podophyllin during the 5th to 9th weeks of pregnancy and gave birth to a baby with multiple deformities.

Toxic antibiotics, which have been considered as anticancer drugs, are generally teratogenic. Actinomycin D has produced congenital malformations in the rat that were polymorphic in nature (Tuchman-Duplessis and Mercier-Parot, 1960). Treatment on the seventh day with actinomycin D produced a significant incidence of malformations (Wilson, 1965). Some of these were types rarely or never seen before. Other teratogenic agents have not produced malformations prior to the eighth day. This early teratogenic effect may be due to the action of this compound. At the enzymatic level synthesis of RNA by DNA-dependent RNA-polymerase is sensitive to the effects of actinomycin D, and this would prevent or interfere with the initial step of the many chemical and morphogenetic steps that are required for the development of any tissue or organ.

Filippi and Mela (1957a) have reported micromelia, ectrodactyly and syndactyly in the offspring of female rats when the dams were given penicillin and streptomycin in doses regarded as equivalent to clinical therapeutic doses. Filippi and

Mela (1957b) have also reported a teratogenic effect of tetracycline in rats when given in long term dosage. A lead article in the British Medical Journal (1965) cautions against the use of tetracyclines in the treatment of pregnant women because of the concentrating of tetracyclines in the fetal skeleton, reduced growth, and a possible connection to malformations.

Another positive teratogen, trypan blue, was described by Gillman et al. in 1948. It produced hydrocephalus, spina bifida and other anomalies in the young of treated rats. It still is a favorite teratogen; but its mode of action remains unexplained.

Goldman and Yakovac (1963) found that maternal immobilization enhanced teratogenicity of salicylate in the pregnant rat. Congenital malformations were produced using subcutaneous injections of sodium salicylate. Immobilization, by itself, produced a marked reduction of fetal growth. However, immobilization markedly increased the production of congenital anomalies by single injections of toxic teratogenic doses of sodium salicylate on the tenth day of gestation in proportion to the dose of salicylate and duration of immobilization.

A maternal deficiency of many of the vitamins has resulted in congenital malformations in the offspring (Kalter and Warkany, 1959). Some of the early work in teratogenesis was based on dietary deficiencies. In 1940 Warkany and Nelson reported that a maternal nutritional deficiency in rats produced

congenital malformations in offspring. Warkany and Schraffenberger (1943) showed that a dietary deficiency of riboflavin was the causative factor. A substantiation of the fact that these malformations were due to riboflavin deficiency was provided by the work of Nelson et al. (1956). These workers used the riboflavin-antimetabolite, galactoflavin, with an increase in the incidence of abnormal young.

Evans et al. (1951) demonstrated that multiple congenital malformations in fetuses of rats resulted when the maternal diet was made acutely deficient in folic acid using x-methyl-pteroylglutamic acid, a crude folic acid antagonist.

Warkany et al. (1959) reported on the use in a human of aminopterin (4-amino-pteroylglutamic acid), a folic acid antagonist, in an attempted abortion. The procedure was not successful and resulted in the birth of a malformed infant.

A maternal deficiency of vitamin $B_{12}$ has been shown to result in hydrocephalus in rat fetuses (O'Dell et al., 1951).

Boisselot as cited in Kalter and Warkany (1959, p. 79) fed a purified diet deficient in pantothenic acid to female rats which resulted in various congenital malformations in the young.

Pinsky and Fraser (1960) reported that 6-aminonicotinamide, a potent nicotinic acid antagonist, produced congenital skeletal anomalies in the young of pregnant mice that had been subjected to a two-hour inactivation of nicotinamide by 6-aminonicotinamide.

A deficiency of vitamin E has been reported to produce fetal abnormalities. Shute (1936) reported hydrocephalus in offspring of female rats kept on a vitamin E-free diet. Frank congenital malformations of various types due to vitamin E deficiency have been reported by Cheng and Thomas (1953). However, Gortner and Ekwurtzel (1965) reported that they could not duplicate the high congenital malformation rate reported by Cheng and Thomas (1953) obtained by administering a tocopherol supplement on the 9th to 11th day of gestation to vitamin E-restricted rats.

A rachitogenic diet given to young female rats and continued throughout pregnancy has produced skeletal defects in the offspring (Warkany, 1943).

Runner and Miller (1956) have reported that a 24 or 30-hour fast of a certain strain of mice during the 7th to 10th days after conception produced congenital malformations in the young. This finding seems to be without precedent among vertebrates. Runner (1959) has produced evidence in mice which showed that very similar deformities result from the use on the ninth day of either folic acid antagonist, iodoacetate, fasting or insulin. He suggested that all these agents interfere in the citric acid cycle, probably at different levels.

Nucleic acid antagonists have produced teratogenic effects in laboratory animals. Thiersch (1957) gave antimetabolites of purine to rats on various days of gestation and produced malformations in the fetuses. Murphy and Karnofsky (1956)

used azaserine, a glutamine antagonist which is an inhibitor of purine biosynthesis, to produce skeletal malformation in rat fetuses. Murphy (1960) reported on the teratogenic effect of a number of tumor-inhibiting chemicals in the fetal rat. The tumor-inhibiting chemicals used were chlorambucil, aminopterin, 6-mercaptopurine, azaserine, 6-diazo-5-oxo-1-norleucine (DON), 5-fluoro-2-deoxyuridine (FUDR), 6-aminonicotinamide, thiadiazole, and triazene. In some cases, metabolites were injected following a dose of an antimetabolite to attempt prevention of toxic effects on the fetuses.

Excessive vitamin A intake during pregnancy has been shown to produce congenital malformations. Cohlan (1953) was the first to show this effect. He used rats as an experimental animal.

The impetus to research in the area of teratogenesis came with the thalidomide incident. Some of the first reports associating thalidomide with congenital abnormalities in human babies were by McBride (1961) and Lenz (1962). Mellin and Katzenstein (1962) have written a thorough review covering the thalidomide literature to late 1962. The varying teratogenic response of different strains and species of laboratory animals to thalidomide became evident. This explained the inability to detect the teratogenic action of thalidomide (Cahan, 1964).

Little work in experimental teratology has involved the effect of agents on the male and subsequent congenital mal-

formations in offspring. Lukwak-Mann (1964) described deleterious effects on offspring of male rabbits that were treated with thalidomide and subsequently mated to does of high fertility 2 to 10 weeks from the end of thalidomide administration. Deleterious effects on the progeny ascribable to paternal treatment with thalidomide were reduced litter numbers, smaller litters and one male offspring in each of two litters by the same male that showed gross malformations.

Numerous reports have appeared concerning the experimental production of congenital malformations by various means (Cahan, 1964; Karnofsky, 1965; Kalter and Warkany, 1959). The interest in this research is emphasized by the mass of information on the teratogenic effects of pharmaceutically interesting compounds. Woollam (1965) lists 120 different drugs known to produce death or malformation, or both in experimental animals.

## Deficiency of Vitamin A and Teratogenesis

Experimental mammalian teratology began with Hale's work. His initial report appeared in 1933 concerning results he had obtained with a female pig fed a vitamin A-deficient diet. A litter of 11 was born, all of which exhibited anophthalmia and two of which had ectopic kidneys. The sire was bred to two other females on normal rations and normal offspring resulted. In 1935 and 1937 Hale reported further results. Using the same general procedure but different sires, he reported the occurrence of anophthalmia, microphthalmia, harelip, cleft palate,

accessory ears, otocleisis, malformed hind legs, undescended kidneys, ectopic ovaries, cryptorchidism and subcutaneous cysts. These abnormalities did not all appear in any one individual but occurred in various combinations in the offspring. He raised malformed individuals from these litters on adequate rations and then mated a blind male to a normal unrelated gilt, to its dam, and to a blind sister littermate with the result that no abnormal pigs were farrowed. With this series of reports Hale demonstrated that a deficiency of a nutritional factor could induce congenital malformations and that genetic factors were not responsible for the malformations observed.

Anderson (1941, 1949) demonstrated that a genetic predisposition to diaphragmatic hernia in her strain of rats was manifested to a much greater degree in the absence of vitamin A.

Warkany and Schraffenberger (1944, 1946) reported congenital malformations of the eyes of young rats that were the offspring of vitamin A-deficient females. Female rats were raised on a vitamin A-deficient diet which contained small measured amounts of carotene. The carotene was necessary for growth and maturation of the females but insufficient to provide vitamin A for storage. At sexual maturity the females were given a diet entirely free of vitamin A and carotene. While on this diet the females were bred and kept throughout pregnancy. In one series of experiments only 7 of 140 females

carried their offspring to term and 39 young were obtained. All of the eyes that were sectioned were found to be abnormal. The most constant abnormal finding was a retrolenticular membrane in place of the vitreous body. Coloboma, abnormal structure and folding of the retina, anomalies of the iris and "open eyes" were some of the other anomalies reported. Jackson and Kinsey (1946) confirmed these findings. They also reported a serum vitamin A level below 12 I.U. per 100 ml. in all of the mothers that produced young with abnormal eyes. Jackson and Kinsey concluded "that the ocular defects occur in the young rat only when the maternal vitamin A deficiency is extremely severe, so advanced in fact, that fetal resorption is common and normal birth is impossible."

Wilson and Barch (1949) confirmed the experiments of Warkany and co-workers using two strains of rats. These workers used three criteria for recognition of imminent death and resorption of conceptuses, thereby making it possible to collect many severe malformed fetuses that would otherwise be destroyed <u>in utero</u>. These criteria were: 1) the presence of more than 25 per cent cornified cells in the vaginal smear at any time during pregnancy; 2) the presence of blood in the vaginal smear in excess of or at times other than that comprising the usual placental sign; or 3) the loss of weight by the mother after the tenth day of pregnancy.

Warkany and Roth (1948) found that increasing the amount of carotene added to the preparatory diet increased the fertility of the females but decreased the incidence of abnormal

young. They recommended that it was advisable to supplement the preparatory diet with 12 to 25 mcg. carotene every tenth day. When early termination of pregnancy threatened, the animal was opened and the fetuses removed for examination. The eyes of at least one animal in each litter were sectioned serially. If no retrolenticular membrane was found, the animal was considered normal, but, if a membrane was seen in any section, the animal was considered abnormal and the litter of origin of that animal was considered abnormal. Wilson and Warkany (1950) had never found any other anomaly existing in the absence of ocular malformations. In addition to ocular malformations, anomalies of the soft tissues were found. These were: undeveloped lungs, spongy heart muscle, diaphragmatic hernia, renal hypoplasia, undistended renal pelves and ureters, horseshoe kidneys, undescended testes and rarely agenesis of one lung and tracheoesophageal fistula (Warkany et al., 1948). Some of these young also had cardiovascular malformations, which were analyzed by Wilson and Warkany (1949). These consisted of defects in the interventricular septum and in the aortico-pulmonary system, a trabeculated and spongy myocardium, and various aortic arch anomalies such as supernumerary, absent, ectopically arising and transposed vessels. It was concluded that all the malformations were the result of interference with normal processes occurring on or subsequent to the 12th day of gestation. Many of the cardiovascular anoma-

lies observed in rats closely simulated malformations observed in man (Wilson and Warkany, 1950).

In addition to the above mentioned renal anomalies, many other malformations of the genito-urinary tract were discovered in histological studies (Wilson and Warkany, 1948). These genito-urinary anomalies were classified on the basis of the apparent manner in which normal developmental processes had been altered in order to produce the observed abnormality. The four categories which seemed to cover all types of malformations were (1) paraplasia, resulting from aberrant processes, (2) aplasia, complete absence of a structure ordinarily present, (3) hypoplasia, resulting from arrested or slowed processes, and (4) hyperplasia, resulting from over-activity of a process. A large majority of these malformations appeared to have been the result of hypoplasia (Wilson and Warkany, 1948).

Keratinizing metaplasia was found by Wilson and Warkany (1947) in epithelia derived from the urogenital sinus but was never found in any fetus prior to the 18th day. This would indicate that the embryo and fetus produce a different histologic response to vitamin A deficiency from that seen in the animal after birth.

The broad array of congenital malformations observed in the abnormal offspring of vitamin A-deficient female rats has been referred to as a "syndrome" (Warkany et al., 1948). Over 9 per cent had both ocular and urogenital anomalies, over 50 per cent had diaphragmatic hernia, and 17 per cent had cardio-

vascular malformations. This syndrome could be modified by administration of a single dose of vitamin A (16,000 I.U. orally) to pregnant females at various times during pregnancy (Wilson et al., 1953). The following conclusions were drawn from this study: (1) Administration of a single dose of vitamin A progressively reduced the incidence of malformed young the earlier given in gestation. (2) The syndrome of malformations was modified by vitamin supplementation on particular days, both as to type of defect and relative percentage of various defects. This was interpreted as being the result of salvaging young that would have otherwise perished. (3) The malformations resulting from maternal vitamin A deficiency were determined during the period of active organ formation rather than on undifferentiated tissues.

Rokkones (1955) noted hydrocephalus in the offspring of vitamin A-deficient rats, although Warkany's group did not observe this. Millen et al. (1954) reported congenital hydrocephalus in the offspring of female rabbits fed a carotene-free diet 14 to 38 weeks before mating. When the carotene-free diet was fed 14 weeks before mating, the offspring manifested hydrocephalus at 2 to 8 weeks after birth (Millen et al., 1953). The hydrocephalic young had well-marked stenosis of the cerebral aqueduct at the level of the superior colliculi. Constriction of the optic nerves was seen. The hydrocephalus was considered to be responsible for the constriction. Overgrowth of the bones of the skull was not evident. Evidence suggested

that the determining factor for hydrocephalus may be over-production of cerebrospinal fluid with a relative insufficiency of the cerebral aqueduct. Millen and Dickson (1957) found increased cerebrospinal fluid pressures in hydrocephalic young, and noted that administration of 10,000 to 20,000 I.U. of vitamin A given weekly starting at one week of age to affected young, produced a steady fall in pressure, which reached normal limits within 2 to 3 weeks.

Lamming et al. (1954) observed ocular malformations in the offspring of rabbits fed a carotene-free diet 12 to 14 weeks before mating. These ocular defects appeared similar to the "open eye" condition reported by Warkany and co-workers (1944, 1946, 1948).

## Effects of Dietary Nitrate and Nitrite on Vitamin A

By using in vitro procedures Pugh et al. (1962) reported on the destruction of β-carotene by $KNO_2$ with various molar ratios and a range of pH values. Destruction of β-carotene by nitrite was greatest at a pH of 1 through 3. Vitamin A alcohol was destroyed at higher molar ratios of nitrite to substrate at pH 2.

Olson et al. (1963) reported that β-carotene is not affected by nitrate under a variety of conditions in an in vitro situation. In an acid medium, nitrite caused rapid β-carotene destruction.

Bloomfield et al. (1962a) reported on the concentration of nitrate in the stomach of rats following intraperitoneal injection of $NaNO_3$. A gastric juice:plasma ratio of 20:1 was reported at low plasma concentrations. The presence of gastric nitrite in the nitrate injected animals was sporadic and could not be quantitated. Kearley et al. (1962) reported that in the wether there was a gastric concentration of intravenously administered nitrate.

Roberts and Sell (1963) reported on the effect of $KNO_2$ on the destruction of vitamin A in abomasal fluid from sheep and ventriculus fluid from chickens in an in vitro situation. When the pH was below 4, almost complete destruction of the vitamin A was observed by the end of a 60-minute incubation period. When 2 per cent $KNO_2$ was fed to fistulated sheep, no nitrite was found in either rumen or abomasal fluid at approximately 90 minutes after feeding. Furthermore, there was relatively little difference in vitamin A concentration between abomasal fluids collected from sheep receiving the control and nitrite rations, suggesting that the dietary nitrite did not enhance vitamin A destruction in the sheep. However, when broilers were fed 0.74 per cent $KNO_2$, nitrite was found in the ventriculus and intestine two hours after feeding, and, in addition, the concentration of vitamin A in these areas was much reduced compared with control birds.

Mitchell et al. (1965) reported that in steers with abomasal fistulas nitrate had no effect on reducing the vitamin A

recovered in the abomasum although extensive ruminal loss was evident in both control and $KNO_3$ treatments.

Reddy and Thomas (1962) reported on the interrelationship between thyroid status and nitrate on *in vitro* carotene conversion to vitamin A. Their results indicated that in hypothyroidism conversion of carotene to vitamin A was reduced. The presence of nitrate in the medium reduced conversion of carotene to vitamin A whether the duodenal homogenate originated from a control, a hypo- or hyperthyroid animal.

Jordan *et al.* (1963) reported that beef cattle fed corn silage of varying nitrate content showed increased blood levels of carotene as a result of intramuscular administration of triiodothyronine. Triiodothyronine increased liver vitamin A in animals receiving supplemental vitamin A or carotene, and increased blood levels of vitamin A in unsupplemented animals.

Moore *et al.* (1965) observed that nitrate added to a ration for calves had no effect on the utilization of carotene from corn silage. Davison *et al.* (1962) reported that nitrate ingestion in cattle had no effect on liver storage of vitamin A. Hale *et al.* (1961) reported that 1 per cent $KNO_3$ in the ration of steers caused a trend in reduced liver vitamin A stores but the difference from controls was not significant.

Jordan *et al.* (1961) reported that liver vitamin A levels of steers on a full feed of silage which had different nitrate levels were reduced greatest on the higher nitrate silage after approximately 70 days. At the end of 133 days, the

liver vitamin A values in all lots were reduced to the same low level.

Koch et al. (1963) reported that $NaNO_2$ in the diet of swine reduced liver vitamin A stores as did dietary $NaNO_3$.

Goodrich et al. (1962) reported on the effects of 3 per cent dietary $NaNO_3$ on the vitamin A status of sheep. Sodium nitrate, with or without the addition of vitamin A, resulted in a significant lowering of liver vitamin A stores. An effect on plasma vitamin A values by the nitrate was uncertain.

Goodrich et al. (1964) reported that in sheep receiving added nitrate to the ration (3.5 and 3.0 per cent) lower liver vitamin A levels were produced. Hatfield et al. (1961) reported that liver stores of vitamin A were lowered in all groups of lambs fed high nitrate silage vs. normal silage.

Sokolowski et al. (1961) reported on the utilization of dietary $KNO_3$ by lambs as affected by inorganic sulfur. No statistically significant differences were found in thyroid, adrenal or pituitary weights, or liver vitamin A. The addition of inorganic sulfate to give 0.5 to 0.9 per cent sulfur in the ration appeared to aid in the prevention of toxic effects of $KNO_3$ by increasing the utilization of the nitrate nitrogen by the growing-fattening lamb. Cline et al. (1962) also reported that $KNO_3$ had no effect on liver vitamin A levels in lambs. Davison et al. (1963) reported on the effects of dietary nitrate in sheep. Plasma vitamin A and liver vitamin A did not reflect the varying nitrate concentrations of the

diets. Smith et al. (1962) reported that in steers and sheep $KNO_3$ did not exert a significant effect on liver vitamin A values.

Holst et al. (1961) reported on the addition of nitrite to the ration of sheep. There was loss of an undetermined amount of nitrite between the time that it was added to the diet and the time that the feed was consumed by the sheep. Rumen microorganisms reduced 80 per cent of the nitrite within 15 minutes. Liver vitamin A was low in the animals fed nitrite but no statement was made as to its significance as compared with control animals.

Sell and Roberts (1963) described the effects of dietary nitrite on the chick. Nitrite (0.4 per cent dietary nitrite as $KNO_2$) depressed growth under ad libitum feeding conditions, but, when feed intake was equalized, supplemental vitamin A or carotene almost completely overcame the growth depressing effect of nitrite. Liver vitamin A levels were very low in chicks fed the various levels of vitamin A or carotene along with nitrite. In all instances dietary nitrite caused a greater loss of ration vitamin A than of injected vitamin A.

Sodium nitrite administered continuously in the drinking water of growing swine for a 105-day period had no adverse effect on liver vitamin A stores (Seerley et al., 1965). Vitamin A values were higher from livers of pigs on the highest level of nitrite than from livers of control animals ($17.4 \pm 3.9$ and $14.4 \pm 2.5$ mcg. of vitamin A/gm. of liver, respec-

tively). Liver vitamin A content of pigs from nitrate-treated dams receiving 300 parts per million (p.p.m.) nitrate nitrogen was not adversely affected by the nitrate treatment (Seerley et al., 1965).

Garner et al. (1958) reported on the effect of adding $KNO_3$ to the ration of bred sows 35 days after breeding. At a 2 per cent level of $KNO_3$ one litter showed evidence of a vitamin A deficiency. Viability and number of strong pigs appeared to decrease with higher levels of nitrate. By using weanling rats, the depletion of liver vitamin A was studied. The depletion appeared to be more rapid with $NO_3^-$ and $NO_2^-$ containing rations. Histologic studies failed to reveal a true vitamin A deficiency in spite of a deficiency syndrome.

O'Dell et al. (1960) reported on the effects of nitrite containing rations in rats. By using a vitamin A-deficient diet, it took 6 to 8 weeks to deplete liver vitamin A. Rats that had received 0.3 per cent $KNO_2$ in the vitamin A-deficient diet showed a greater reduction in growth rate, and liver vitamin A correlated with the growth data in the two groups. Rats that had been vitamin A depleted were then subdivided so that each group had subgroups receiving $\beta$-carotene or $\beta$-carotene and 0.3 per cent $KNO_2$. Again growth was depressed by $KNO_2$. After four weeks with carotene added the rats in the original $KNO_2$ group showed a dystrophy typical of vitamin E deficiency. Not only did nitrite cause rapid depletion of vitamin A, but it also precipitated a vitamin E deficiency on

a diet normally adequate in vitamin E. Appetite and muscular coordination improved when vitamin E concentrate was given to the rats in this group.

Tollett et al. (1960) reported on the effect of dietary nitrate on growth and reproduction in swine. High levels of nitrate depressed gains significantly, and supplemental vitamin A did not counteract the reduction in gains. High levels of nitrate increased the methemoglobin level. Growth of gilts was depressed by high levels of nitrate (3.17 per cent), but reproductive ability was not affected nor was thyroid weight affected.

Yadov et al. (1962) described the relation of iodine and nitrate as factors in vitamin A storage. Male rats were placed on vitamin A-deficient rations; (1) low iodine (0.2 mcg./gm.), (2) low iodine-nitrate (1.5 per cent $KNO_3$), (3) adequate iodine (2 mcg./gm.) and (4) adequate iodine-nitrate, until they were vitamin A deficient. Analysis revealed that nitrate reduced liver vitamin A. Adequate iodine in the diet resulted in a reduced loss when compared with inadequate iodine. Remaining rats were repleted with 1 mg. $\beta$-carotene in the diet for four weeks. After the repletion period rats receiving adequate iodine stored much greater vitamin A per liver than those receiving low iodine. Those receiving nitrate at the two iodine levels stored a lesser amount of liver vitamin A than those not receiving nitrate.

Smith et al. (1961) reported that dietary nitrite in rats resulted in decreased liver vitamin A stores. Potassium nitrite was added to the diet at 0.8 per cent dry basis and fed for 25 days.

Emerick and Olson (1962) found that 0.5 per cent sodium nitrite fed for six days to vitamin A-depleted rats decreased the liver storage of orally administered vitamin A and β-carotene when compared with that stored by control rats or rats receiving 3 per cent $NaNO_3$ in the diet. Injection of vitamin A in oil resulted in very small amounts of vitamin A appearing in the livers of the rats fed the control and nitrite diets. Storage of the injected dose was significantly higher in the rats receiving nitrite, but was unexplained. In all instances the water dispersed source of vitamin A contributed to liver storage to a much greater extent than the oil solution and the orally administered sources to a much greater extent than the injected sources. Both nitrite and nitrate significantly lowered the liver storage of vitamin A from carotene with the greatest effect resulting from nitrite. The six-day nitrite treatment did not result in less liver vitamin A than controls when neither group received any vitamin A, although values were very low in each case.

Effects of Dietary Nitrate and Nitrite on the Thyroid

Wyngaarden et al. (1952) studied the ability of 20 different anions, including nitrate and nitrite, to interfere with

normal iodine metabolism of the thyroid gland of rats fed a low iodine diet. Perchlorate was the most potent and nitrate the least potent as compared with thiocyanate in discharging iodide previously collected by the thyroid. The results with nitrite were equivocal in that initial discharge was not significant but the subsequent loss of radio-iodide exceeded the spontaneous loss in control groups. Capacity to prevent collection of iodide by the thyroid approximately paralleled their iodide discharging action. Nitrite was not tested in this manner. Rats fed nitrate for a period of time developed only minor degrees of hyperplasia and slight reduction of thyroidal iodine concentrations; whereas, rats treated with perchlorates developed hyperplastic colloid-depleted low iodine goiters.

Astwood (1943) reported on the relative effectiveness of a number of compounds in inhibiting the function of the thyroid in young rats. The administration of sufficient inorganic iodide prevented the goitrogenic effects of thiocyanates but not that of thiourea.

Franklin et al. (1944) demonstrated that thiocyanate interferred with thyroidal accumulation of iodine and the formation of thyroxine and diiodotyrosine in contrast to thiouracil which blocked the organic incorporation of iodine but did not inhibit its collection by the thyroid.

Bloomfield et al. (1961) reported that rats on a diet containing $KNO_3$ at concentrations of 0.5, 1.0 and 2.5 per cent

for six days showed a significant reduction in thyroidal uptake of $I^{131}$ at all levels of nitrate. Sheep fed a diet containing 1.5 per cent $KNO_3$ for six days showed a marked reduction in protein bound $I^{131}$. Bloomfield et al. (1962c) reported that rats on a diet containing 2.5 per cent $KNO_3$ in a normal environment initially showed a decreased thyroidal $I^{131}$ uptake but were able to overcome this effect after two weeks. The flushing action of the $KNO_3$ on the $I^{131}$ from the thyroid was not overcome. Thyroid gland weights in the nitrate group were larger than those in the control group. Serum protein bound $I^{131}$ was higher in the $KNO_3$ group than in the controls. The increased size of the thyroid glands was also reported for young rats fed 2.5 per cent $KNO_3$ for 75 days and for mature female rats fed a diet containing 2.5 per cent $KNO_3$ for 30 days (Welsch et al., 1961). The diet contained 72 p.p.m. as a calculated iodine level. Adrenal glands were larger in the nitrate group, but the differences were not significant.

Gordon et al. (1965) reported on the chronic effects of $NH_4NO_3$ on iodine balance in rats. Nitrate initially increased urinary iodide excretion and decreased fecal iodide excretion. After seven days on nitrate total thyroid iodine was reduced to 60 per cent of its control and urinary and fecal excretion of iodide gradually returned to the control values. The plasma protein bound iodine was 76 per cent greater in the nitrate rats than in the controls after 110 days on experiment.

$I^{131}$ uptake was reduced, release was increased, and the thyroids were enlarged in the animals fed nitrate.

Welsch et al. (1962) reported on rats that were on a diet with 72 parts per billion (p.p.b.) of iodine with 2.5 per cent $KNO_3$ added and maintained at 2°C. The thyroid glands were significantly enlarged and hyperplastic in the nitrate group. When the experiment was repeated using a diet with a calculated iodine level of 1081 p.p.m. there was no significant difference in thyroid gland weight or activity as determined histologically. However, the rate of gain of the nitrate group remained significantly less than that of the control group.

Bloomfield et al. (1965) concluded that 200 p.p.b. of iodide were sufficient to overcome 2.5 per cent dietary $KNO_3$ in rats maintained at 2°C. for 35 days.

Sell and Roberts (1963) reported that dietary nitrite in the chick produced thyroid gland hypertrophy which was most severe when low levels of vitamin A were given. Increasing the level of vitamin A or β-carotene reduced the thyroid gland enlargement somewhat, but the nitrite still produced a very enlarged thyroid that was approximately twice the size of the control group at each level of vitamin A or β-carotene.

Bloomfield et al. (1962b) reported on thyroidal $I^{131}$ metabolism in sheep fed a basal diet plus 1.5 per cent $KNO_3$ for six days prior to injection of $I^{131}$. Six-day thyroidal uptake of $I^{131}$ was significantly higher in the nitrate group than in the controls, whereas serum protein bound $I^{131}$ was

reduced. However, 27 days after injection the serum protein bound $I^{131}$ was significantly greater in the nitrate group than in the controls.

Jainudeen et al. (1965) found that feeding nitrate to pregnant heifers at levels to 660 mg. nitrate ion/kg. of body weight had no effect on thyroid gland weights, acinar cell heights and pituitary thyroid stimulating hormone content. Davison et al. (1962) had not observed thyroid abnormalities in cattle fed nitrate.

Hypothyroidism and teratogenesis

The literature includes case reports in which hypothyreosis of the mother is claimed to be the cause of congenital defects in the child. Elphinstone (1953) reported on an infant whose mother received methylthiouracil after the first 16 weeks of pregnancy that had hypothyroidism at birth. The infant recovered from the hypothyroidism but subsequent mental development was retarded. Literature concerning other cases was reviewed. Morris (1953) described a clinical case of hypothroidism in an infant whose mother had been treated with methylthiouracil after the first 14 weeks of pregnancy. Thyroid therapy was given for ten days after birth. When eight months old the infant showed signs of hydrocephalus. A neurosurgeon suggested that there was probably stenosis of the aqueduct. Hodges et al. (1952) described the offspring of a myxedematous woman that had had the condition for 15 years.

Four children had survived and were found to be euthyroid. The children displayed congenital defects: moronism, mongolism, a clawfoot, double canine tooth and undescended testis. These suggested that the myxedematous mother provided an inadequate environment for normal growth and development of her fetuses.

Hoet et al. (1959, 1960) described and discussed pregnancy wastage (congenital malformations, miscarriages and stillbirths) in the human. Treatment with insulin or thyroid extract was successful in preventing pregnancy wastage in a selected group with mild endocrine disturbances. Hypovitaminosis A, based on clinical signs such as carotinadermia and night blindness which are common symptoms of prediabetes and hypothyroidism, was proposed as one of the prenatal causes of malformations in the human newborn. They proposed that the hypovitaminosis A was due to a reduced transformation of provitamins to active vitamin A. It was suggested that the genesis of active vitamin A at the level of the effector organ was disturbed and deficient under such adverse metabolic circumstances as diabetes and hypothyroidism. This same relationship between the endocrinopathies and active vitamin A metabolism may be relevant at the level of the fetus and placenta. In selected patients with night blindness insulin or thyroid treatment corrected the night blindness in five days.

Administration of goitrogenic agents to pregnant laboratory animals has been reported. Most investigators made no

report of congenital malformations being produced.

Krementz et al. (1957) reported on the effect of administration of propylthiouracil to pregnant rabbits starting day 11 of pregnancy. Fetal thyroids showed a significant relative weight gain. There was a definite decrease in average fetal body weight when the dam received propylthiouracil during pregnancy. No malformations were reported.

Freiesleben and Kjerulf-Jensen (1947) found only slight transitory hyperplasia in the thyroids of fetuses of rats fed methylthiouracil throughout pregnancy. This disappeared if the drug was discontinued immediately after birth. Administration of thyroid hormone to the female with the methylthiouracil prevented the fetal thyroid hyperplasia. Nikitovitch and Knobil (1955) fed propylthiouracil to rats from the 12th day of pregnancy. They found a highly significant increase in fetal thyroid weight which was not modified or prevented by prior hypophysectomy of the mother. They concluded that maternal thyroid stimulating hormone does not cross the placental barrier and that the increase in fetal thyroid weight is mediated by the fetal pituitary-thyroid axis. Malformations were not reported.

Krohn and White (1950) reported on the effect of hypothyroidism in the female rat. Rats were injected with propylthiouracil for at least 54 days before mating or were thyroidectomized at least 28 days before mating. Reproductive ability

was compared to that achieved by these same animals in a previous normal pregnancy. The induced hypothyroid state produced estrous cycles that were longer and more variable than normal. Fewer young were brought to term than in the previous pregnancies. This was due to increased resorption of fetuses in the hypothyroid state. Malformations were not observed.

Nelson and Tobin (1937) reported that thyroidectomy of female rats during the last half of pregnancy or prior to pregnancy resulted in the delivery and suckling of normal litters.

Reports in the literature of hypothyroidism as a cause of defects of the fetuses are few. Chu as cited in Krohn (1950, p. viii) noted frequent resorptions and abortions of underdeveloped fetuses when rabbits were thyroidectomized in early pregnancy. Krohn (1950) did not reproduce Chu's results. Normal litters were delivered. Langman and van Faassen (1955) produced malformations in the offspring of Wistar female rats that were partially thyroidectomized 7 to 38 days before, but not 2 to 7 days after, conception. The most frequent abnormalities were those involving the eyes, such as cataractous lenticular changes, coloboma, retinal folding and occasional anophthalmia. Cleft palate, cleft lip, retarded ossification and hemorrhages were also found. Preliminary study by these same investigators with methylthiouracil in the drinking water for some time before

pregnancy produced eye defects of the same nature as those obtained by thyroidectomy. None of these defects were found in control offspring.

Stempak (1962) was unable to confirm the data of Langman and van Faassen. He used Holtzman strain rats and did a partial thyroidectomy or a complete thyro-parathyroidectomy prior to pregnancy. Defects in experimental and control litters were comparable.

## Effects of Dietary Nitrate and Nitrite on Reproduction

Weeds containing nitrates were reported to cause abortion in cattle (Sund and Wright, 1957). Feeding of $KNO_3$ and $NaNO_2$ to cattle produced the same placental lesions that were noticed in natural occurring cases. These lesions were attributed to a tissue anoxia caused by methemoglobinemia.

Muhrer et al. (1956) reported on the effect of $KNO_3$ on reproduction in rats. All 12 control rats produced normal litters. Nine of 12 animals produced litters on a 1 per cent $KNO_3$ ration. Two out of 8 animals produced litters on a 2 per cent $KNO_3$ ration. It was observed that four of these animals produced placental tissue but did not produce litters indicating that the fetuses had been absorbed or aborted. They also reported that $KNO_3$ produced a marked drop in milk production in the bovine.

Welsch et al. (1965) investigated the previous finding of Muhrer's group at Missouri on the effect of dietary nitrate on reproduction of the rat. Chronic administration of 2.5 per cent $KNO_3$ to males and females through several pregnancies did not interfere with normal reproductive processes. Histopathologic evaluation of neonatal thyroid tissue showed no significant effect of nitrate, and no apparent differences in thyroid gland size were noted.

Sinclair and Jones (1964) administered $KNO_3$ to pregnant ewes. When the ewes were 49 to 70 days pregnant, they were placed on $KNO_3$ sprayed hay. No abortions occurred but the lambs from the nitrate treated ewes were smaller than those from the control ewes. Davison et al. (1963) found that dietary nitrate had no effect on birth weight of lambs.

Davison et al. (1964) reported that the conception rate in heifers receiving 660 mg. nitrate ion/kg. of body weight was lowered. However, growth, length of estrous cycle, length of gestation, birth weight and performance of calves were similar in all groups.

Sodium nitrate (300 p.p.m. nitrate nitrogen) given continuously in the drinking water to swine from weaning through two farrowings had no adverse effects on litter size, birth weights, daily gain or litter size at weaning (Seerley et al., 1965).

## Methemoglobin Formation and Dietary Nitrate and Nitrite

A survey of nitrate levels in well waters revealed quite a variation in nitrate nitrogen. Water supplies with levels of 70 to 130 p.p.m. of nitrate nitrogen were located near soils contaminated with organic nitrogen by livestock (Schmidt, 1956). Walton (1951) surveyed the literature relative to infant methemoglobinemia and nitrate-contaminated water. Most cases of methemoglobinemia were associated with nitrate nitrogen concentration in excess of 40 p.p.m.

Zobell (1932) demonstrated in vitro that many organisms commonly found in the gastrointestinal tract of man are capable of reducing nitrates to nitrites. In a review of literature Comley (1945) mentioned that van den Bergh and Gutterinck reported in 1906 that nitrites could be formed in the intestines from nitrates and that the nitrite was more likely to be absorbed if the intestine was damaged.

Conant (1933) noted that the reaction of nitrite with hemoglobin to form methemoglobin was an oxidation reaction in which the ferrous iron in hemoglobin was oxidized to ferric iron in methemoglobin. Methemoglobin is unable to transport oxygen. Ordinarily this reaction is reversible and occurs without permanent injury to the cells. Cyanosis characterized by greyish or brownish-blue coloration is attributed to the abnormal pigment, methemoglobin, within the red cell. Five

grams of reduced hemoglobin/100 ml. of blood are required to produce recognizable cyanosis, whereas 1.5 gm. of methemoglobin/100 ml. of blood has comparable effects (Finch, 1948).

At concentrations of 20 per cent methemoglobin working subjects complained of fatigue and showed abnormally high blood lactic acid levels (Tepperman et al., 1946). Lester and Greenberg (1944) reported that dogs and cats could tolerate more than 80 per cent conversion of hemoglobin to methemoglobin without becoming unconscious.

The red cell reduces methemoglobin by an enzymatic process in which glucose and lactate are the principal substrates (Finch, 1948). The presence of methemoglobin activates this process since resting oxygen consumption and glucose utilization of the red cell are only slight, whereas in the presence of methemoglobin there is an appreciable increase. It appears that hexosemonophosphate and reduced nicotinamide adenine dinucleotide (NADH) diaphorase are involved in this reaction (Fialkow et al., 1965). Production of methemoglobinemia can be caused either by dysfunction of the reconversion mechanism or by the action of oxidants which produce methemoglobin more rapidly than the cell mechanism is able to reduce it.

Nitrite is much more toxic for rats than is nitrate. Wanntorp and Swahn (1953) reported that the $LD_{50}$ of sodium nitrate for female rats weighing 150 to 200 gm. was 5 gm./kg. of body weight. The $LD_{50}$ for sodium nitrite was determined to be 90 mg./kg. of body weight. From these experiments it

appeared that the toxicity of nitrite was approximately 50 times as high as that of nitrate. Methemoglobin was not found in the blood of rats that received nitrate. This would indicate that nitrate was not reduced to nitrite in the digestive tract of rats. With sub-lethal doses of nitrite the methemoglobin concentration of the blood of rats reached its maximum as early as 1 to 1.5 hours after administration. The concentration then fell slowly and after 4 to 6 hours there was no measurable methemoglobin in the blood.

Mortensen (1953) showed in rats that the intraperitoneal administration of 28 mg. sodium nitrite/kg. of body weight resulted in an average methemoglobin level of 4.05 gm./100 ml. of blood one hour after administration. Three hours after administration it was 0.55 gm./100 ml. of blood.

Wang et al. (1961) reported on metabolism of nitrate by cattle. Nitrate was rapidly reduced into nitrite and ammonium ions in the rumen. The formation of methemoglobin followed the time course of nitrite formation in the rumen rather closely, suggesting that nitrite was passed rapidly and directly from the rumen into the blood. Maximum concentration of nitrite in the rumen appeared about three hours after nitrate was added to the rumen, and the maximum conversion of hemoglobin to methemoglobin occurred at almost the same time.

Lewis (1951) reported that nitrate introduced into the rumen of the sheep was reduced to ammonia. Nitrite was an intermediate in this reaction and may accumulate under certain

conditions and lead to a conversion of hemoglobin to methemoglobin. Sodium nitrate (25 gm.) or sodium nitrite (10 gm.) placed in the rumen or 2 gm. of sodium nitrite injected intravenously resulted in a methemoglobinemia corresponding to 60 per cent conversion of the total hemoglobin.

Stormorken (1953) presented data concerning methemoglobinemia in domestic animals. He found that for cattle the minimum oral lethal dose of sodium nitrite in aqueous solution was about 100 mg./kg. of body weight. The response in sheep was similar to that of cattle. Swine were more sensitive to sodium nitrite than cattle and sheep. The minimum lethal dose of sodium nitrite in this species was 70 to 75 mg./kg. of body weight. In cattle it was found that methemoglobin disappeared at the rate of 9 per cent per hour following a single treatment.

Davison et al. (1962) reported on the effect of high nitrate intake in cattle. Blood hemoglobin tended to increase following feeding of nitrate, but there was also a trend in all animals toward decreasing hemoglobin levels with advancing pregnancy. Much variation was evident in production of methemoglobin, but methemoglobin production paralleled nitrate ingestion. Packed cell volumes (PCV) paralleled hemoglobin. The results indicated an apparent adaptation of the erythropoietic system of animals fed nitrate to the reduced oxygen-carrying capacity of the blood.

Nitrate fed to pregnant heifers at the rate of 20 or 30 gm./100 lb. of body weight per day resulted in an erythropoetic response. Hemoglobin, methemoglobin, PCV, erythrocyte count and blood volume were significantly greater in the nitrate-fed animals than in the controls (Jainudeen et al., 1963).

Smith et al. (1962) reported that only methemoglobin values were affected in steers fed nitrate.

Bloomfield et al. (1962b) found that feeding 1.5 per cent $KNO_3$ to sheep produced a change in hemoglobin values. Twelve-day hemoglobin (gm./100 ml.) values were determined and values for the nitrate fed group were significantly higher than for the control group (11.93±0.23 and 10.83±0.47, respectively). Holst et al. (1961) reported that the addition of nitrite to the ration of sheep did not result in an increase in methemoglobin. Rumen microorganisms reduced 80 per cent of the nitrite within 15 minutes. Goodrich et al. (1962) reported low methemoglobin values in sheep that received 3 per cent $NaNO_3$ in their diet. Goodrich et al. (1964) again reported small amounts of methemoglobin at regular bleedings in sheep receiving 2.5 and 3 per cent $NaNO_3$. However, seven sheep died from suspected acute nitrate toxicity while being fed 3 per cent $NaNO_3$.

Sokolowski et al. (1960) described the effects of varied levels of $KNO_3$ ingested by lambs. Levels of $KNO_3$ to 12.8 per cent of the ration were fed without causing any visible

symptoms of nitrate toxicity. A subsequent drenching of the lambs with 64 gm. of $KNO_3$ resulted in symptoms of extreme nitrate toxicity within four and one-half hours after drenching, and methemoglobin levels were 70 per cent of total hemoglobin by the fourth hour. In a different trial administration of $KNO_3$ by capsules resulted in toxicity symptoms while $KNO_3$ in a complete ration produced no apparent toxicity effects. Sokolowski et al. (1961) reported that the addition of inorganic sulfate to the ration of lambs receiving high levels of nitrate to give 0.5 to 0.9 per cent sulfur in the ration aided in the prevention of toxic effects of $KNO_3$ by increasing utilization of the nitrate nitrogen. Methemoglobin values were normal. Smith et al. (1962) found that $KNO_3$ had no effect upon hemoglobin, methemoglobin and hematocrit values. Davison et al. (1963) found that hemoglobin and methemoglobin did not reflect varying concentrations of nitrate in the diet of sheep.

Sinclair and Jones (1964) reported on the effect of nitrate administration to sheep. Drenching once daily with $KNO_3$ resulted in a very slight rise in methemoglobin at the end of the first week. Subsequently the methemoglobin concentration returned to normal. Administration of $KNO_3$ on the hay at a level of 1.5 per cent of dry matter did not produce any change in methemoglobin levels. No changes in hemoglobin or PCV were produced.

Seerley et al. (1965) reported that sodium nitrite administered continuously in the drinking water of swine did not

result in much methemoglobin production. The highest average value observed was 0.47±0.10 gm. methemoglobin/100 ml. of blood at a level of 100 p.p.m. nitrite nitrogen. All groups had decreasing methemoglobin levels at each succeeding sampling period.

Anoxia and congenital malformations

A simulated atmospheric pressure of 260 mm. Hg. or more for five hours at different stages of pregnancy produced fetal death or congenital deformity in mice (Ingalls et al., 1950). Anoxia produced on the 9th, 10th and 11th day of pregnancy produced a marked decrease in number of embryos.

Ingalls et al. (1952) reported again on anoxia in mice and its effects on fetuses. Distribution of embryos in verified pregnancies according to location in right or left horn of the uterus was analyzed. No finding emerged to indicate that one side provides a more favorable location for implantation and maintenance, even under stress, than the other. A variety of congenital defects were noted.

Fernandez-Cano (1958) described the effect of increased or decreased body temperature and hypoxia on pregnancy in the rat. An increase or a decrease of body temperature of rats for five hours for two consecutive days during early pregnancy was found to induce embryonic resorptions mainly before implantation. Hypoxia at 410 mm. Hg. of barometric pressure induced embryonic degeneration mainly after implantation especially

when applied on days 10 and 11 of pregnancy. Malformations were not noted.

Woollam and Millen (1961) reported that in the mouse effects of anoxia from reduced barometric pressure were greatest on the fetuses that implanted at the vaginal end of the horn. Resorptions and stunting occurred most frequently at these implantation sites.

Chicken eggs incubated to 40 hours in an air mixture of 6 or 8 per cent $O_2$, 0.5 per cent $CO_2$ and the remainder $N_2$ experienced a high degree of maldevelopment which frequently involved the alar-plate tissues resulting in pseudoencephaly (Nelsen, 1960).

Curley and Ingalls (1957) kept pregnant mice for two hours during the tenth day of gestation in a chamber through which was directed a mixture of nitrogen and air containing 6 per cent $O_2$ at normal atmospheric pressure. Skeletal malformations were produced. This was the first report to reveal that in mice exposure of the mother to an atmosphere of normal pressure with reduced oxygen tension can induce vertebral and rib anomalies in the young. Previous experiments that used hypoxia as a stressing agent had been carried out in such a manner that the animals also exhibited lowered body temperature and also were without nutriment during the experimental period. Results then were controversial.

Haring and Polli (1957) exposed female rats for 3 to 12 days at various times during pregnancy to a gas mixture containing increased $CO_2$ with normal or decreased $O_2$ tension in the respiratory atmosphere. Offspring were killed between term and nine days of age. The treatment increased the number of nonviable offspring at birth. Myocardial thickening and some septal defects were noted in sections of hearts.

Brent and Franklin (1960) reported on the use of uterine vascular clamping to produce malformations, growth retardation and fetal death in nine-day rat embryos. The technique consisted of placing a hemostatic clamp at the cervical and ovarian ends of one uterine horn for a predetermined period of time, removing the clamps and examining embryos at a later stage of gestation. Franklin and Brent (1964) studied the effect of uterine vascular clamping on the development of rat embryos. There was no difference in uterine sites as regards resorption rates.

McLaren and Michie (1960) in a discussion of congenital runts in mice stated that there was a significant association between runting and the ovarian position in the uterine horn. Placental fusion with another live embryo also showed significant association with runting. The ovarian embryo often shares its blood supply with the ovary, and the fused embryo shares its blood supply with its fused partner. They suggested that the factor involved in runting may be reduced blood supply. A correlation between incidence of cleft lip and cleft palate

and occupancy of the ovarian position in the uterine horn was cited. The role of limitation of blood supply might provide a common basis for uniting congenital runting with congenital malformations that can be induced by reduced oxygen tensions (Ingalls et al., 1952).

Wilson (1954) reported on the influence on offspring of altered physiologic states during pregnancy in the rat. He used hemorrhagic anemia, carbon tetrachloride poisoning, trypan blue injections and vitamin A deficiency as maternal stresses. There was little correlation between the severity of the maternal reaction, as reflected in maternal death or early termination of pregnancy, and the extent to which offspring were affected in surviving litters. It was postulated that those agents capable of acting on or through the pregnant mother to cause maldevelopment of the young must have some specific relation to the needs of the developing embryo rather than have non-specific influences that happen to act at a critical time in development.

Muller and Graham (1955) reviewed a number of cases of carbon monoxide poisoning during pregnancy in humans. Anatomic anomalies and psychomotor disturbances in the offspring were described. There was not complete agreement among authors reviewed as to whether carbon monoxide can enter fetal circulation or not. Effects seen may be due to simple fetal hypoxia at a critical time in development.

# MATERIALS AND METHODS-EXPERIMENT I

## Experimental Design

### Animals

A Sprague-Dawley rat colony[1] maintained by the Department of Physiology and Pharmacology was used as the source of experimental animals. The rats were individually ear notched for identification at weaning time, 25 days of age. Four experimental female animals were in a 10" x 16" cage except when exposed to males until the females were zero days pregnant at which time two animals were placed in an 8" x 10" cage. All experimental animals were not available at the same time.

### Division of groups

Four treatment groups were established on the basis of diet as follows:

I. Control diet

II. Control diet plus 0.75 per cent $KNO_2$ (1.25 mg. nitrite nitrogen/gm. of feed)

III. Vitamin A-free diet

IV. Vitamin A-free diet plus 0.75 per cent $KNO_2$ (1.25 mg. nitrite nitrogen/gm. of feed)

Animals were randomly distributed to their groups at the time that they were determined to be zero days pregnant.

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[1]Holtzman Co., Madison, Wisconsin.

Unequal group sizes were used for a total of 94 animals. Group sizes were as follows: group I, 22; group II, 31; group III, 19; and group IV, 22.

Method of handling

When the female rats reached a body weight of at least 180 grams, they were mated. Two male rats were placed in a cage of four or less females at 5:00 P.M. and removed in the morning. A vaginal smear was made daily from each female exposed to the males in order to confirm breeding. Day zero of pregnancy was the day that sperm were found in the vaginal smear.

Rats comprising groups I and II were fed a commercial laboratory ration.[1] The ration was fed finely ground throughout the period of pregnancy. Potassium nitrite (0.75 per cent) was mixed with the ground ration daily and the animals were fed newly prepared feed once a day. Paired feeding was conducted between rats in groups I and II and between rats in groups III and IV. Groups III and IV were fed a purified vitamin A-free diet from 30 days of age (Table 1). Forty I.U. of vitamin A[2] in an aqueous solution were administered orally with a cannula to each rat every 10 days. When the female rats were approximately breeding size, severe symptoms of

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[1]LAB-BLOX, Allied Mills, Chicago, Illinois.

[2]Palmilets, supplied by Chas. Pfizer and Co., Inc., New York, New York.

vitamin A deficiency became apparent and deaths from respiratory infections occurred. The dosage of vitamin A was increased to 80 I.U. and was given to each rat every 10 days. Vitamin A was not given during pregnancy. The drinking water was changed three times a week.

Table 1. Composition of vitamin A-free diet

| | % |
|---|---|
| Casein (devitaminized)[a] | 20.0 |
| Dextrose | 64.9 |
| Corn oil | 5.0 |
| Cellulose | 5.0 |
| Mineral mix[b] | 5.0 |
| Vitamin mix[c] | 0.1 |
| | 100.0 |

[a]Sheffield Chemical, Norwich, New York.

[b]Jones and Foster (1942). NaCl, 13.9%; $KH_2PO_4$, 38.9%; $MgSO_4$, 5.73%; $CaCO_3$, 38.1%; $FeSO_4.7H_2O$, 2.7%; KI, 0.08%; $MnSO_4.2H_2O$, 0.44%; $ZnCl_2$, 0.26%; $CuSO_4.5H_2O$, 0.048% and $CoCl_2.6H_2O$, 0.002%.

[c]Thiamine-HCl, 0.5%; Riboflavin, 0.8%; Niacin, 4.0%; Pyridoxine, 0.5%; Ca-Pantothenate, 4.0%; Biotin, 0.04%, Folic acid, 0.2%; Menadione, 0.5%; Cyanocobalamin ($B_{12}$), 0.003%; Inositol, 10.0%; p-Amino Benzoic Acid, 10.0%; alpha-Tocopherol Succinate, 2.2%; vitamin $D_2$, 0.4% (500 I.U./mg.); Dextrose, q.s. 100%.

## Body Weight Analyses

Initial body weights were recorded at day zero of pregnancy. The final body weights were recorded at day 20 of pregnancy. All of the final body weights were used in calcu-

lations whether the females were pregnant or not.

## Blood Analyses

Blood samples were obtained by cardiac puncture under light ether anesthesia on days 5, 12 and 20 of pregnancy. Clotting was prevented by the use of disodium ethylenediaminetetraacetate (EDTA) powder.

The PCV was determined by the microhematocrit method. Whole blood was drawn into heparinized microhematocrit tubes and centrifuged for five minutes at approximately 11,500 r.p.m. The percentage of packed cells was determined by reading directly from a microhematocrit reader. Hemoglobin values were obtained by the cyanmethemoglobin[1] method. Methemoglobin values were obtained by the method of Evelyn and Malloy (1938) immediately after collection of the sample.

## Small Organ Weights Analyses

The females were killed on day 20 of pregnancy in a jar saturated with ether. The pituitary gland and adrenal glands with adjacent adipose tissue were removed and put into Bouin's solution. The upper trachea with the thyroid gland attached was removed and put into 10 per cent formalin solution.

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[1]Hycel Cyanmethemoglobin Determination Instructions. Hycel, Inc., Houston, Texas.

Twenty-four hours later the small organs were isolated from any extraneous tissue, placed on paper toweling, weighed 10 minutes later to the nearest 0.1 mg. and stored in 70 per cent alcohol.

## Liver Analyses

The liver was the first organ removed from the female. It was blotted with paper toweling, weighed, stored under nitrogen in plastic bags that were heat-sealed and immediately placed in a deep freeze at -20°C.

Liver vitamin A analyses[1] were made at a later time by a modified Carr-Price method (Gallup and Hoefer, 1946). Equal aliquots of liver (1 gram) were taken from the frozen livers to form composite samples for each treatment group.

## Fetal Analyses

Prior to death a Cesarean section was quickly performed on the females for the purpose of examination of the uteri and contents. The number of live fetuses per litter and the weight of the litter were recorded. The average weight per fetus in a litter was utilized in the calculations.

The number, position and duration of resorptions were recorded. The distribution of position and duration of re-

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[1]Analyses were conducted by M. Ruffin and B. Symns, Home Economics Nutrition Laboratory, Iowa State University, Ames, Iowa.

sorptions were analyzed by chi-square (Snedecor and Cochran, 1956). Early resorptions showed yellow nodular implantation remnants in the uterus at delivery. Late resorptions usually showed only placentas present at delivery, but at times fetuses in the process of resorption were evident.

Fetuses were initially put into Bouin's solution and later transferred to 70 per cent alcohol for storage. One fetus from each litter was used as the source of material for sectioning. The fetuses were examined grossly. The heads were removed as was the lower jaw by a cut with a razor blade through the commissures of the lips and continuing posteriorly. The anterior portion of the muzzle was also removed. Paraffin tissue blocks of the heads were prepared.

Sections were made using a standard microtome with section thickness of 40 microns. Every eighth section was mounted on glass slides except at the level of the eyes where every fourth section was utilized. The sections were stained with hematoxylin and eosin (Armed Forces Institute of Pathology, 1957).

Fetuses were examined grossly for malformation at the time that the fetuses were removed from the female. When the heads were prepared for sectioning, gross examination of the fetuses was again conducted. Microscopic examination of the sections was conducted. Emphasis was directed toward the detection of malformations of the eyes. Photomicrographs were taken on a Leitz Photomicroscope.

## Data Analyses

Data for this research project were assembled and processed with a computer.[1] The variables studied were as follows: initial body weight, final body weight, hemoglobin, methemoglobin, PCV, liver weight, average fetus weight, number of fetuses, number of resorptions and weights of adrenals, thyroid, and pituitary. The data were analyzed at the 5 and 1 per cent levels of significance by an analysis of variance, correlation coefficients (Snedecor and Cochran, 1956) and a modification of Duncan's multiple range test (Kramer, 1965).

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[1]Computation Center, Iowa State University, Ames, Iowa.

# RESULTS AND DISCUSSION-EXPERIMENT I

## Body Weight Analyses

The initial body weights averaged 216, 211, 202 and 196 grams for group I, control; group II, control plus nitrite; group III, vitamin A-free; and group IV, vitamin A-free plus nitrite, respectively (Figure 1). Unless otherwise indicated, values for treatments will be given in the above order throughout the experiment. The differences among treatment means were significant at the 1 per cent level. The multiple range test (5 per cent level) indicated that the significant differences were between group I (control group) and the vitamin A-free groups (I vs. III or IV). Variability in initial body weights can be attributed to differences in the length of time that ensued between the exposure to the males and day zero of pregnancy.

The mean final body weights were 285, 264, 247, and 216 grams, respectively (Figure 1). The differences among treatment means were significant at the 1 per cent level. The multiple range test indicated a significant difference (5 per cent level) between the means of all possible pairs of groups except groups II vs. III. Paired feeding had been conducted between animals in groups I and II and animals in groups III and IV. Feed requirements were severely reduced in the nitrite groups when the animals were initially placed on treatment. An undetermined amount of treated feed was wasted due to un-

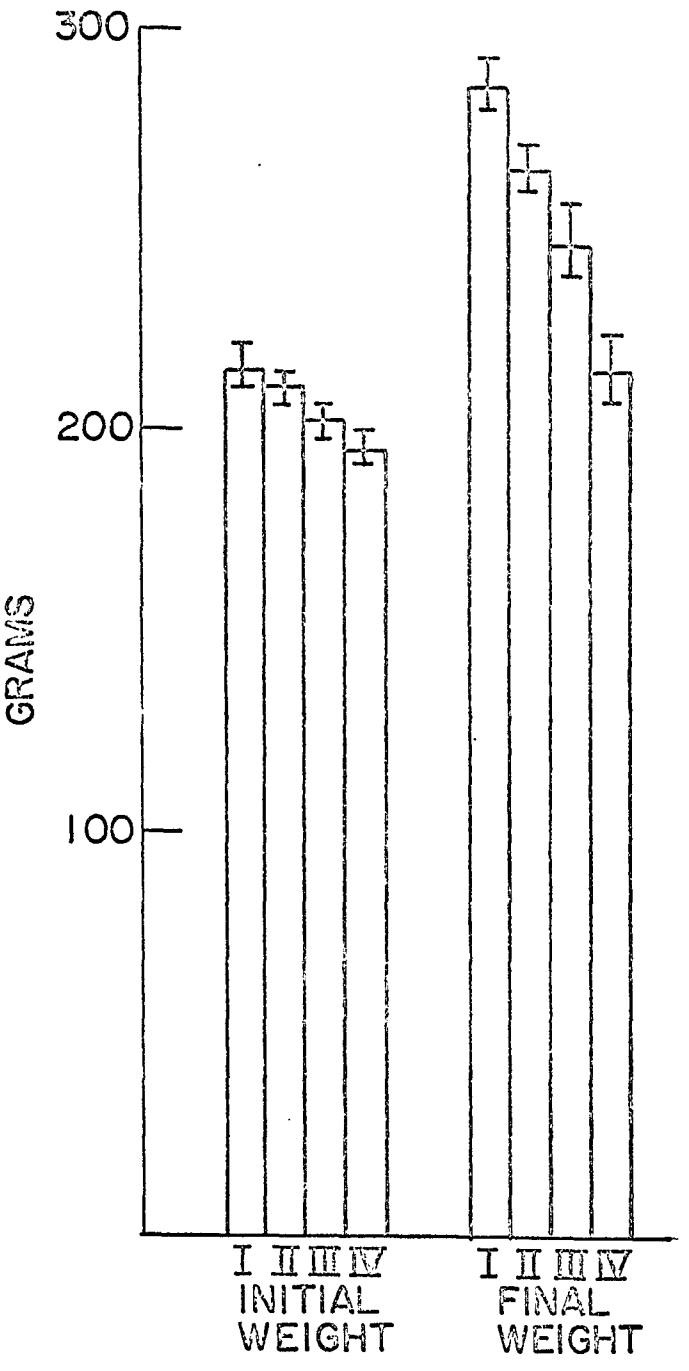


Figure 1. Initial and final body weights of rats in experiment I

palatability. This was more evident during the first days on experiment. After about eight days feed requirements in the treated groups approached the expected normal. The reduced final body weight in nitrite-treated groups could have been primarily due to reduced feed ingested as the amount wasted could not be determined.

Group IV (vitamin A-free plus nitrite) was the only group with significant negative correlations between the final body weights and weights of the adrenal, thyroid and pituitary glands (1 per cent, 1 per cent and 5 per cent levels, respectively). This would indicate that the treatment (vitamin A-free diet plus nitrite) was of such a nature as to produce an increased weight of these organs while resulting in a decreased body weight on day 20. This would be a characteristic result from a stressing agent. However, there were significant negative correlations between initial body weights and weights of the adrenals and thyroid also in this group (5 per cent level). These were not evident in the other vitamin A-free group (group III).

## Blood Analyses

The day 5 PCV averaged 45.2, 43.7, 42.3 and 43.5 per cent, respectively (Figure 2). There was not a significant difference between treatment groups in the day 5 PCV, nor were the day 5 hemoglobin values affected by the treatments. The average hemoglobin values were 14.8, 14.8, 14.5, and 15.0 gm./100 ml. of blood, respectively (Figure 3).

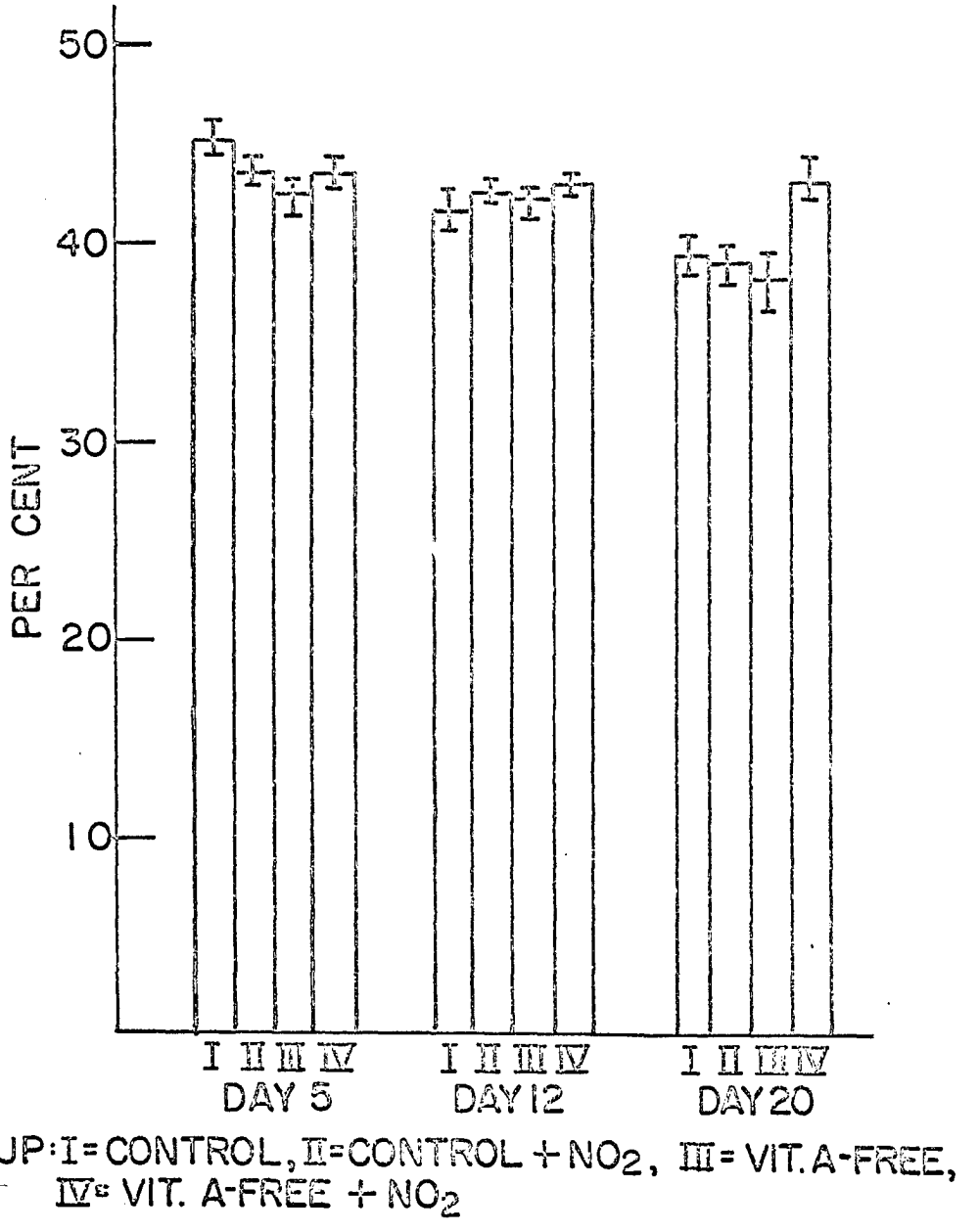


Figure 2. Packed cell volumes (PCV) of rats in experiment I at days 5, 12 and 20

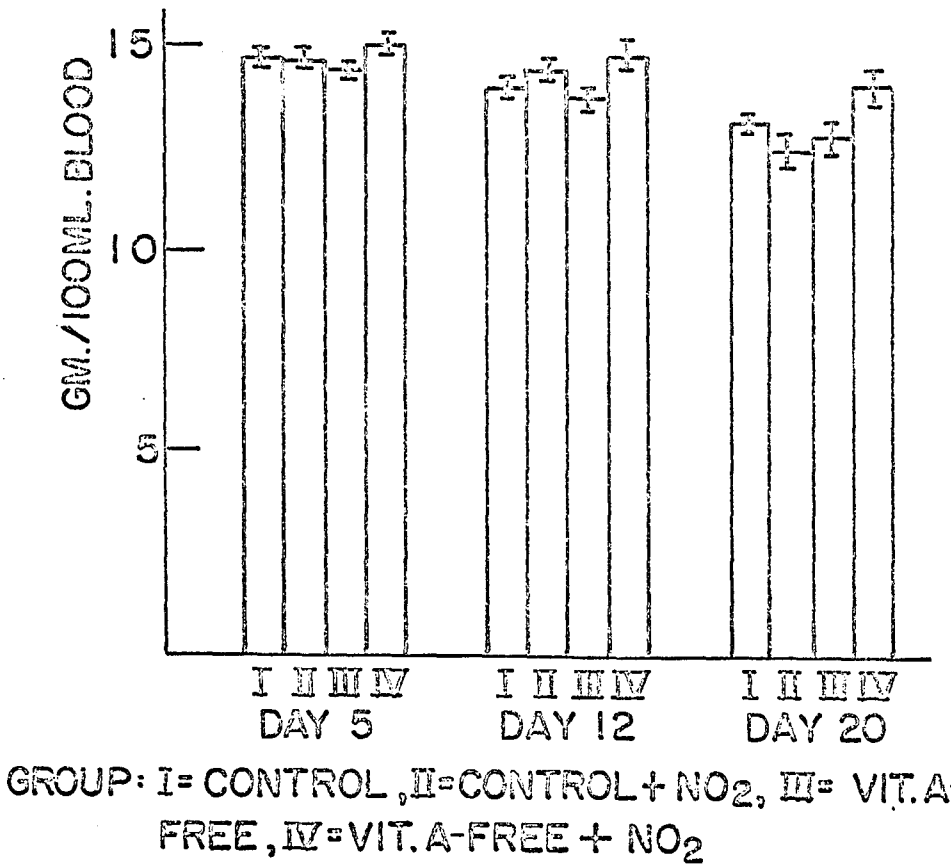


Figure 3. Hemoglobin values of rats in experiment I at days 5, 12 and 20

Methemoglobin values on day 5 averaged 0.18, 0.95, 0.16 and 1.35 gm. per 100 ml. of blood, respectively (Figure 4). The analysis of variance indicated a significant difference between means at the 1 per cent level. The multiple range test (1 per cent level) indicated that the groups receiving nitrite (II and IV) had significantly greater mean methemoglobin values than the groups not receiving nitrite (I and III).

The reaction of nitrite with hemoglobin to form methemoglobin is an oxidation reaction in which the ferrous iron in hemoglobin is oxidized to ferric iron in methemoglobin (Conant, 1933). This reaction is reversible and occurs without permanent injury to the cells (Fialkow et al., 1965). Methemoglobinemia can be caused either by dysfunction of the reconversion mechanism or by the action of oxidants which produce methemoglobin more rapidly than the cell mechanism is able to reduce it. The ingestion of potassium nitrite at a level of 0.75 per cent of the diet evidently resulted in sufficient blood nitrite levels to produce an oxidation of hemoglobin to methemoglobin.

Methemoglobin is unable to transport oxygen. Concentrations of 20 per cent methemoglobin resulted in fatigue of working human subjects (Tepperman et al., 1946). Sufficient methemoglobin can result in an anoxic stimulus to erythropoietic tissue. If methemoglobin levels on day 5 were such as to exert an anoxic stimulus on the erythropoietic tissue,

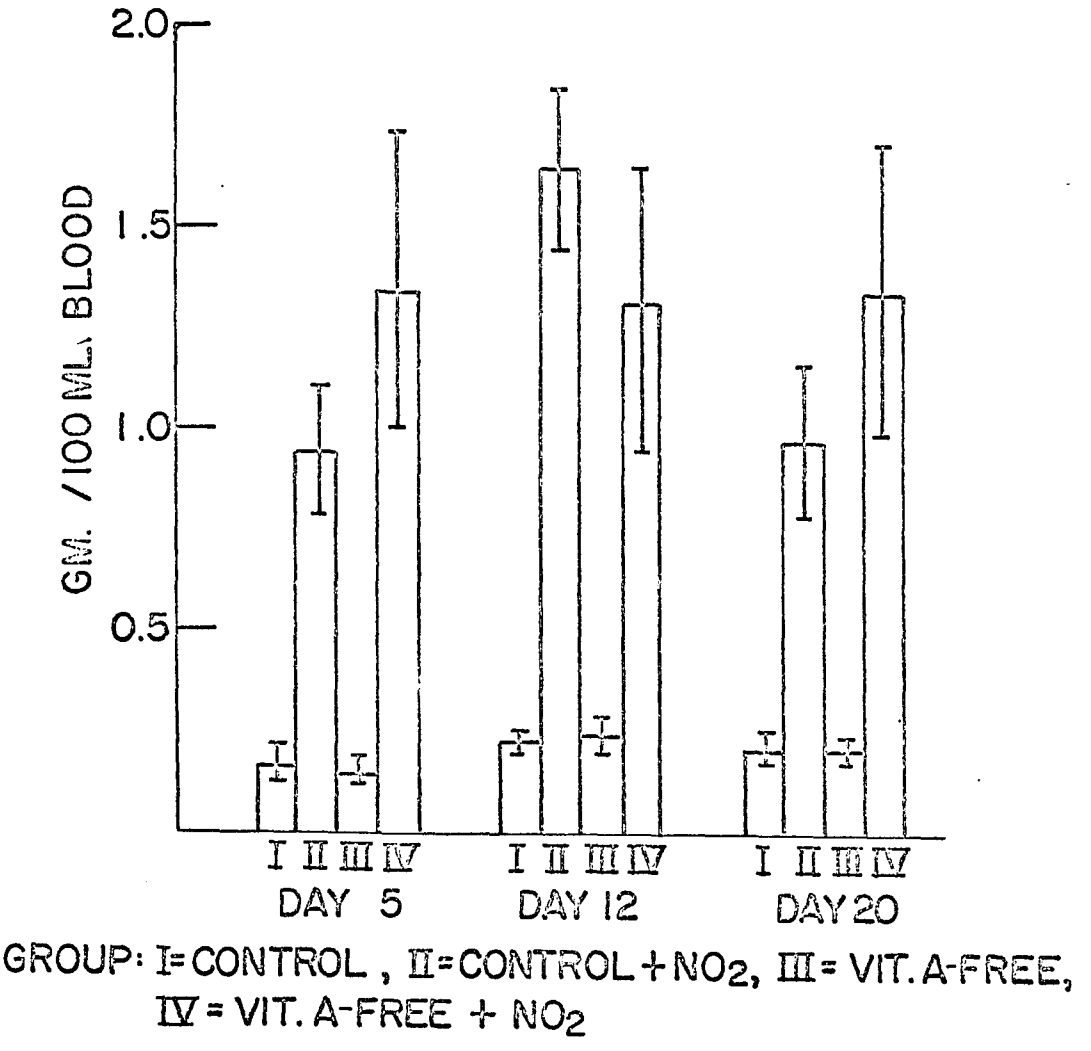


Figure 4. Methemoglobin values of rats in experiment I at days 5, 12 and 20

the effects were not detected in the day 5 PCV and hemoglobin values.

Statistical analysis indicated treatment effects on day 12 hemoglobin values at the 5 per cent level of significance. These mean hemoglobin values were 14.0, 14.5, 13.8 and 14.8 gm./100 ml., respectively (Figure 3). The multiple range test indicated a significant difference at the 1 per cent level between the hemoglobin values of the vitamin A-free and vitamin A-free plus nitrite groups (III vs. IV). The increased hemoglobin in the nitrite groups could be a result of the methemoglobin produced by the potassium nitrite. The presence of methemoglobin could result in chronic anoxic stimulus to the erythropoietic tissue with the resultant increase in hemoglobin that was evident on day 12. Day 12 methemoglobin values were significantly affected (1 per cent level) by the treatments. Mean values of 0.23, 1.66, 0.25 and 1.31 gm. of methemoglobin per 100 ml. of blood were produced (Figure 4). The methemoglobin values reflected the effect of the dietary nitrite in groups II and IV on day 12 as it did on day 5. The multiple range test (1 per cent level) indicated that the groups receiving nitrite (II and IV) had significantly greater mean methemoglobin values than the groups not receiving nitrite (I and III).

PCV on day 20 averaged 39.6, 39.1, 38.4 and 43.4 per cent, respectively (Figure 2). There was a significant difference (5 per cent level) between means of the groups. The multiple

range test (5 per cent level) indicated that the mean PCV of the vitamin A-free plus nitrite group was significantly greater than the other groups (IV vs. I, II or III).

On day 20 mean hemoglobin values were 13.2, 12.6, 13.0 and 14.2 gm./100 ml. of blood, respectively (Figure 4). There was a significant difference between mean values at the 5 per cent level. The multiple range test (5 per cent level) indicated that the vitamin A-free plus nitrite group has a significantly greater mean hemoglobin value than the vitamin A-free and the laboratory ration plus nitrite groups (IV vs. III or II). This could be expected as the PCV was also increased significantly in this group compared with the other groups.

London et al. (1967) reporting on pigs found that the continuous administration of nitrite in the drinking water over a 124-day trial period resulted in increased hemoglobin and PCV values, but no value was above the normal range. Davison et al. (1962) reported increases in mean hemoglobin and PCV values as a result of chronic ingestion of high nitrate in cattle. They reported a trend in all animals toward decreasing hemoglobin and PCV values with advancing pregnancy. In this experiment decreases in mean hemoglobin and PCV values on day 20 compared with values on day 12 were evident in all groups except group IV which had a small increase in PCV on day 20 (Figures 2, 3). The hemodilution effect of advanced pregnancy may have been sufficient to nullify an expected

erythropoietic response in the control plus nitrite group (group II).

Examination of the data revealed positive correlation coefficients (1 per cent level of significance) in all treatment groups between day 20 PCV and hemoglobin. This would be expected. Davison et al. (1962) reported that PCV paralleled hemoglobin in cattle on a nitrate trial.

Hemoglobin and PCV on day 20 in group II (control plus nitrite) did not respond to the nitrite treatment in a manner comparable to group IV (vitamin A-free plus nitrite). This would suggest that in group IV perhaps there may be some cause other than an anoxic stimulus from nitrite for the increased mean hemoglobin and PCV values, or rather, the lack of decrease on day 20.

McLaren et al. (1965) reported that the hemoglobin and PCV values were significantly increased in vitamin A-deficient rats versus rats receiving the same diet but supplemented with four I.U. of vitamin A daily. Sure et al. (1929) had previously reported the hemoconcentration effect of vitamin A-deficiency in the terminal stage of deficiency. Moore (1957) reported that four I.U. of vitamin A daily were sufficient to result in optimal growth and normal blood levels of the vitamin without liver storage. Rats comprising groups III and IV in this experiment received a vitamin A-free diet supplemented with 40 or 80 I.U. of vitamin A every ten days until day zero of pregnancy when no more vitamin A was given. Group

IV then received nitrite in the diet during pregnancy. The dietary nitrite could have produced vitamin A deficiency with a resultant increase in hemoglobin and PCV as reported by Sure et al. (1929) and McLaren et al. (1965). The hemoconcentration effect of vitamin A-deficiency on PCV and hemoglobin may have been sufficient to overcome the hemodilution effect of advanced pregnancy in the vitamin A-free plus nitrite group (group IV).

Garner et al. (1958) reported that nitrite-containing rations fed to weanling rats produced a depletion of vitamin A. Histological studies failed to reveal a true vitamin A deficiency in spite of a deficiency syndrome. O'Dell et al. (1960) found that it took 6 to 8 weeks to deplete liver vitamin A in weanling rats using nitrite-containing rations.

Day 20 mean methemoglobin values were 0.22, 0.98, 0.21 and 1.35 gm./100 ml. of blood, respectively (Figure 4). The mean values were significantly different, and the multiple range test (both at 1 per cent level) indicated that both nitrite groups (II and IV) had greater mean methemoglobin values than the nitrite free groups (I and III). Only group IV (vitamin A-free plus nitrite) had a significant positive correlation (1 per cent level) between day 20 methemoglobin and day 20 PCV and hemoglobin values. Davison et al. (1962) reported that mean methemoglobin values paralleled PCV and hemoglobin values in cattle receiving nitrate.

Mean methemoglobin values on days 5, 12 and 20 reflected the effect of potassium nitrite in the diet, although methemoglobin values were not very large. Methemoglobin values in chronic toxicosis trials may reflect the time interval between nitrite ingestion and sampling. It would appear that methemoglobin levels may be too erratic to serve as a useful diagnostic aid where continuous nitrite intake is involved.

Mortensen (1953) reported that the intraperitoneal administration of 28 mg. of sodium nitrite/kg. of body weight in rats resulted in an average methemoglobin level of 4.05 gm./100 ml. of blood one hour after administration. Three hours after administration there was 0.55 gm. methemoglobin/100 ml. of blood. An ingestion rate for potassium nitrite can be approximated for this experiment. One can consider an average weight of 225 gm./animal during the experimental period, and a mean feed consumption of 20 gm./animal daily. At the level of potassium nitrite in the diet (0.75 per cent or 1.25 mg. nitrite nitrogen/gm. of feed) each rat consumed an average of 110 mg. of nitrite nitrogen/kg. of body weight over a 24-hour period. The ingestion of this amount of nitrite over a 24-hour period would result in a lesser amount of nitrite intake for any one period than that reported by Mortensen and could account for a lower methemoglobin level.

Seerley (1965) reported that 100 p.p.m. of nitrite nitrogen administered continuously as sodium nitrite in the drinking water of swine resulted in mean methemoglobin values of

0.34, 0.47 and 0.17 gm./100 ml. of blood on days 16, 23, and 76, respectively. All treatment groups had decreasing methemoglobin values at each succeeding sampling period except day 23 and were very small at any time.

London et al. (1967) reported that pigs receiving nitrite continuously in drinking water had variable amounts of methemoglobin. Methemoglobin was the highest in pigs receiving 18.3 mg. nitrite nitrogen/kg. of body weight as potassium nitrite, and forty-four per cent of samples in this group contained methemoglobin with an average amount of 8.5 per cent of total hemoglobin existing as methemoglobin. This approximates the amount of total hemoglobin existing as methemoglobin in this experiment.

## Small Organ Weights Analyses

The day 20 adrenal weights averaged 19.2, 22.3, 19.2 and 23.0 mg./100 gm. of body weight, respectively (Figure 5). The differences among treatment means were significant at the 1 per cent level. The multiple range test indicated a significant difference (5 per cent level) between means of the control and control plus nitrite groups (I vs. II) and between means of the vitamin A-free and the vitamin A-free plus nitrite groups (III vs. IV). The feeding of potassium nitrite at the level used (0.75 per cent of the diet) affected the adrenals so that they were significantly larger than those of the non-

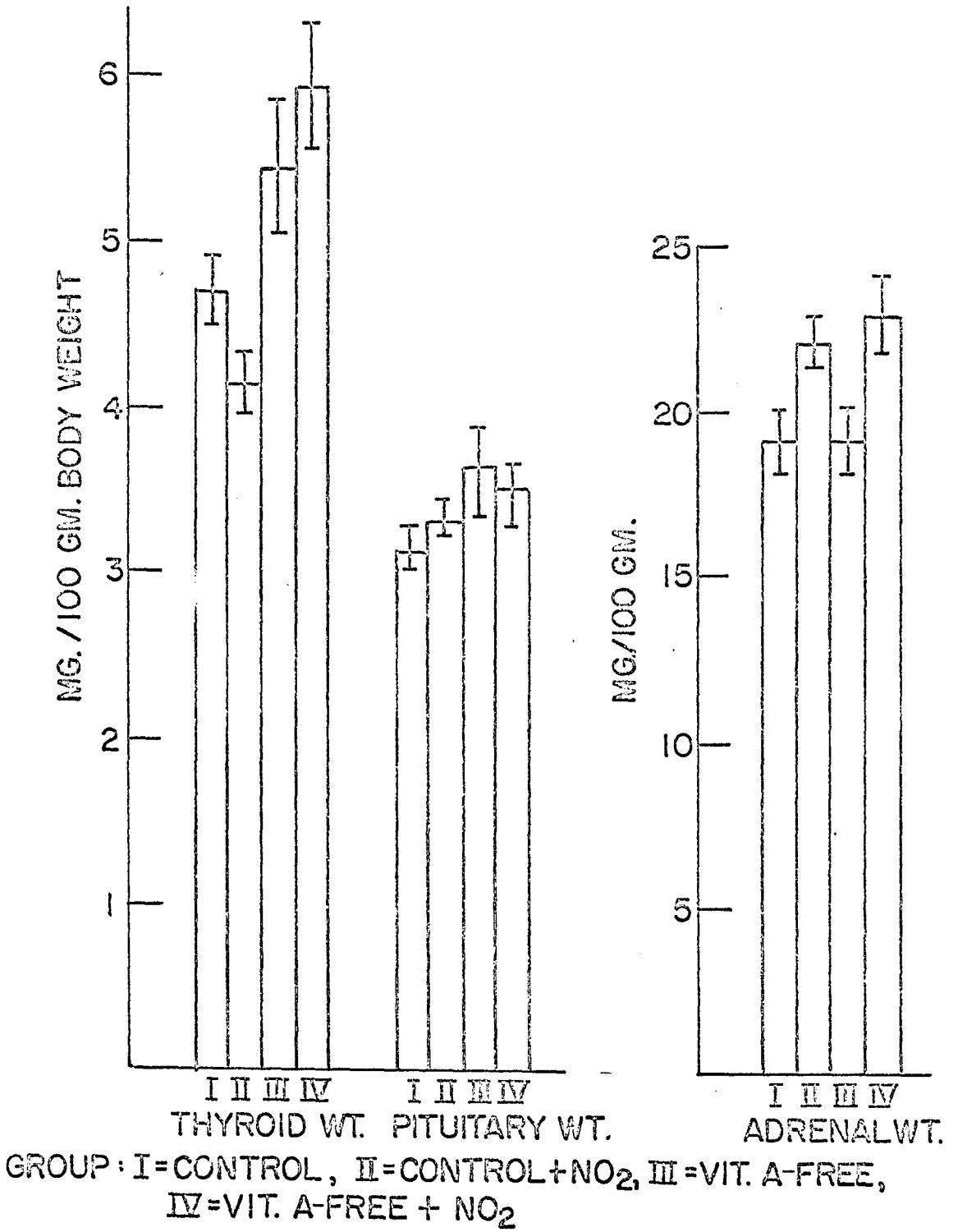


Figure 5. Thyroid, pituitary and adrenal weights of rats in experiment I

nitrite groups. Discussion of the adrenal enlargement in group IV is found in the analyses of body weights.

The day 20 thyroid gland weights averaged 4.7, 4.2, 5.5 and 5.9 mg./100 gm. of body weight, respectively (Figure 5). The differences among treatment means were significant at the 1 per cent level. The multiple range test (1 per cent level) indicated that the differences were not the result of nitrite in the diet. Wyngaarden et al. (1952) reported that in the rat nitrate had a weak effect in interfering with iodide metabolism of the thyroid, and nitrite results were equivocal. Bloomfield et al. (1965) reported that sufficient dietary iodide would overcome the effect of 2.5 per cent dietary $KNO_3$ in rats. Either nitrite at the level used did not cause thyroid enlargement or the diets contained sufficient iodide to overcome any such effect if it did exist.

Jainudeen et al. (1965) reported that the feeding of nitrate, which is reduced to nitrite by rumen bacteria, to pregnant heifers at levels to 660 mg. of nitrate ion/kg. of body weight had no effect on thyroid gland weights, acinar cell heights or pituitary thyroid stimulating hormone content.

The increased mean thyroid weights were associated with the vitamin A-free diet (groups III and IV). Sell and Roberts (1963) reported that dietary nitrite in the chick resulted in hypertrophy of the thyroid gland. Low levels of vitamin A with nitrite produced an increased hypertrophy.

There was a significant (1 per cent level) negative correlation only in group IV between both initial and final body weights and thyroid gland weights. This group also had a significant negative correlation between both adrenal (1 per cent) and pituitary (5 per cent) gland weights and final body weights.

There were no significant treatment differences in the mean pituitary gland weights. These were 3.2, 3.3, 3.7 and 3.5 mg./100 gm. of body weight, respectively (Figure 5). There was a significant positive correlation (5 per cent) between pituitary weights and adrenal weights in the control and vitamin A-free plus nitrite groups (I and IV, respectively). These groups also had the only significant positive correlations between pituitary gland weights and thyroid gland weights (5 per cent level).

## Liver Analyses

The day 20 liver weights averaged 3.6, 3.4, 3.2 and 3.2 gm./100 gm. of body weight (Figure 6). The differences among treatment means were significant at the 1 per cent level. The multiple range test indicated that the dietary nitrite did not have any effect on reducing mean liver weights when comparisons were made between groups receiving the same dietary regimen with the exception of nitrite (I vs. II; III vs. IV).

This may not be in agreement with Emerick and Olson (1962) who reported that 0.5 per cent dietary $NaNO_2$ for six days

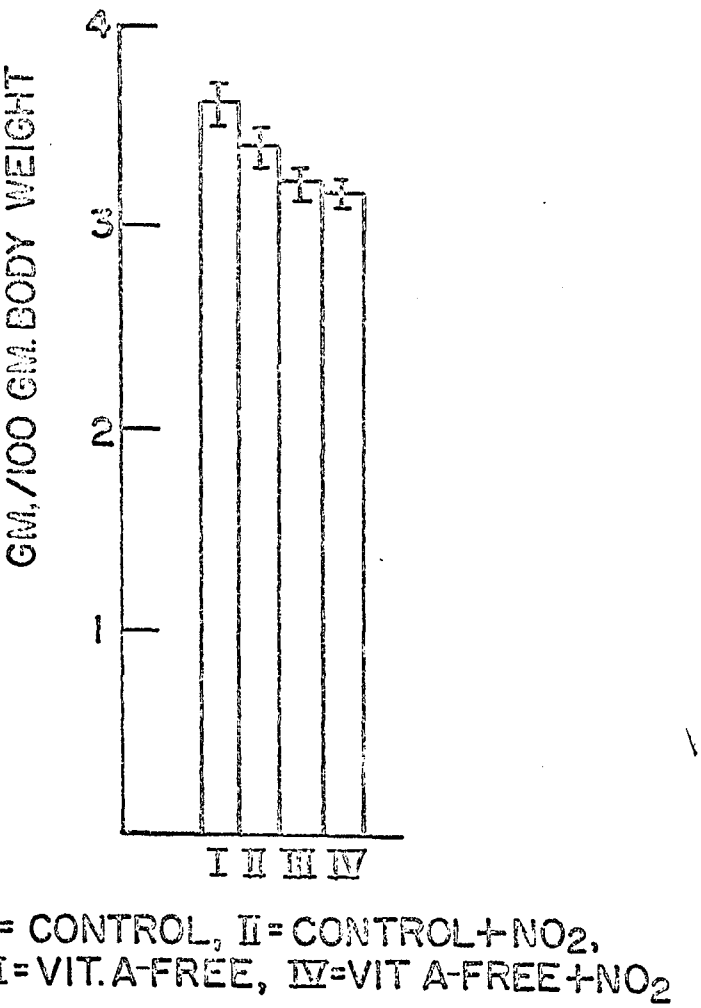


Figure 6. Liver weights of rats in experiment I

reduced liver weights when compared with controls. There may have been sufficient adaptation to nitrite ingestion in this experiment so that no effect on liver size was noted after 20 days on experiment. Methemoglobin values in these rats were low which could be a result of adaptive mechanisms.

Table 2. Liver vitamin A analysis

| | Group and diet | Vitamin A (mcgm./gm. of liver) |
|---|---|---|
| I. | Control | 327.15 |
| II. | Control plus nitrite | 269.94 |
| III. | Vitamin A-free | 10.32 |
| IV. | Vitamin A-free plus nitrite | 14.73 |

Dietary potassium nitrite reduced liver vitamin A when added to the control diet (Table 2). Dietary nitrite in the ration of the vitamin A-free plus nitrite group did not reduce liver vitamin A when compared with the vitamin A-free group. Both values were low when compared with the animals on laboratory ration.

Emerick and Olson (1962) reported that a six day dietary treatment of 0.5 per cent $NaNO_2$ to vitamin A-depleted rats did not reduce liver vitamin A when compared with the vitamin A-depleted rats not receiving nitrite. Values for both groups were low. However, O'Dell et al. (1960) reported that 0.3

per cent dietary $KNO_2$ in a vitamin A-deficient diet depleted liver vitamin A of rats in 6 to 8 weeks. Smith et al. (1961) reported that dietary nitrite resulted in decreased liver vitamin A in rats receiving 0.8 per cent $KNO_2$ for 25 days.

Seerley et al. (1965) reported higher vitamin A values from livers of pigs on continuous dietary nitrite than from livers of control animals (17.4 and 14.4 mcgm./gm. of liver, respectively). Statistical results were not stated.

In this experiment pooled liver samples for vitamin A analysis were formed without regard to the length of time that the rat was on the vitamin A-free diet. If nitrite had an effect on liver vitamin A stores in the rat, this effect may have been masked by the fact that rats in the vitamin A-free group could possibly have been more depleted of vitamin A than those in the nitrite group as a result of being on the vitamin A-free diet for a longer period of time before pregnancy.

## Fetal Analysis

All the females in which sperm were found in the vaginal smear did not become pregnant, nor did all those that became pregnant have viable fetuses at day 20. The incidence of pregnancy in the various treatment groups is summarized in Table 3. The females in the mated row were those found to contain sperm in the vaginal smear and were classified as zero days pregnant at that time. The females in the row designated as numbers with implantations exhibited evidence at day 20 of

Table 3. Reproductive performance of rats in the four treatment groups

| | Group | | | |
|---|---|---|---|---|
| | I Control | II Control $+NO_2$ | III Vitamin A-free | IV Vitamin A-free $+NO_2$ |
| Mated females | 22 | 31 | 19 | 22 |
| No. with implantations | 20 | 23 | 13 | 15 |
| Litters | | | | |
| all alive | 5 | 15 | 7 | 6 |
| 1 resorption | 7 | 5 | 4 | 2 |
| >1 resorption <100% | 8 | 3 | 2 | 3 |
| 100% resorptions | 0 | 0 | 0 | 4 |

having been pregnant by either having live fetuses at that time or by exhibiting resorption sites. Litters were classified into four groups as follows: (1) all alive, (2) one resorption site, (3) more than one resorption site but less than 100 per cent resorptions and (4) 100 per cent resorptions.

By using chi-square analysis and testing for independence of a two-way classification of pregnancy rate, it was found that there were differences in the pregnancy rate due to treatments but only at a rather low level of significance (25 per cent level). A second approach was used to examine the data. This was the chi-square test for goodness of fit which refers to the comparison of some observed sample distribution with a

theoretical frequency distribution. In this case the theoretical frequency was that of pregnant and non-pregnant based upon the control group (group I) with a pregnancy rate of 91 per cent. The chi-square test for goodness of fit indicated that there were significant effect of treatments on pregnancy rate when the theoretical frequency was that of the control group (group I). Pregnancy rates in the other groups (II, III and IV) were reduced when compared with the rate of group I. Group I had a pregnancy rate of 0.91, whereas, the combined rate for the other groups was 0.70. The effect of reduced pregnancy rate can be attributed to the ingestion of nitrite by group II and the decreased levels of vitamin A in groups III and IV.

By using chi-square and testing for independence of a two-way classification there were no effects of treatments on number of females delivering live fetuses without resorptions, number of females with one resorption and number with more than one resorption but less than 100 per cent resorptions. The only treatment group with females exhibiting 100 per cent resorptions of litters was the vitamin A-free plus nitrite group (group IV).

Warkany and Schraffenberger (1944) reporting on congenital malformations induced in rats by maternal vitamin A deficiency stated that in one series of experiments only 7 of 140 females carried their offspring to term. Certainly the animals in that experiment were more deficient in vitamin A than were

those animals reported here.

Muhrer et al. (1956) reporting on the effect of potassium nitrate on reproduction in rats found that a two per cent potassium nitrate ration resulted in only 2 litters out of 8 females. Four animals exhibited evidence of resorptions or abortions. However, Welsch et al. (1965) reported that the chronic administration of 2.5 per cent potassium nitrate to males and females through several pregnancies did not interfere with normal reproductive processes. These reports were concerned with the effects of nitrate while the experiments reported here concerned effects of nitrites.

There was a significant difference (1 per cent level) in the mean number of live fetuses for the different treatments. The mean number of live fetuses were 7.2, 7.2, 4.6 and 3.7 fetuses (Figure 7). The multiple range test (1 per cent level) indicated that dietary nitrite had no significant effect on the mean number of fetuses (III vs. IV). The vitamin A-deficient groups had significantly less fetuses in the litters than the females receiving laboratory ration (III or IV vs. I or II). There was a significant positive correlation in all groups between number of fetuses and final body weight (5 per cent level). This may indicate that the same homeostatic conditions of a female necessary to produce a large number of fetuses may be those necessary for a successful body growth, or it could be a result of experimental design as the

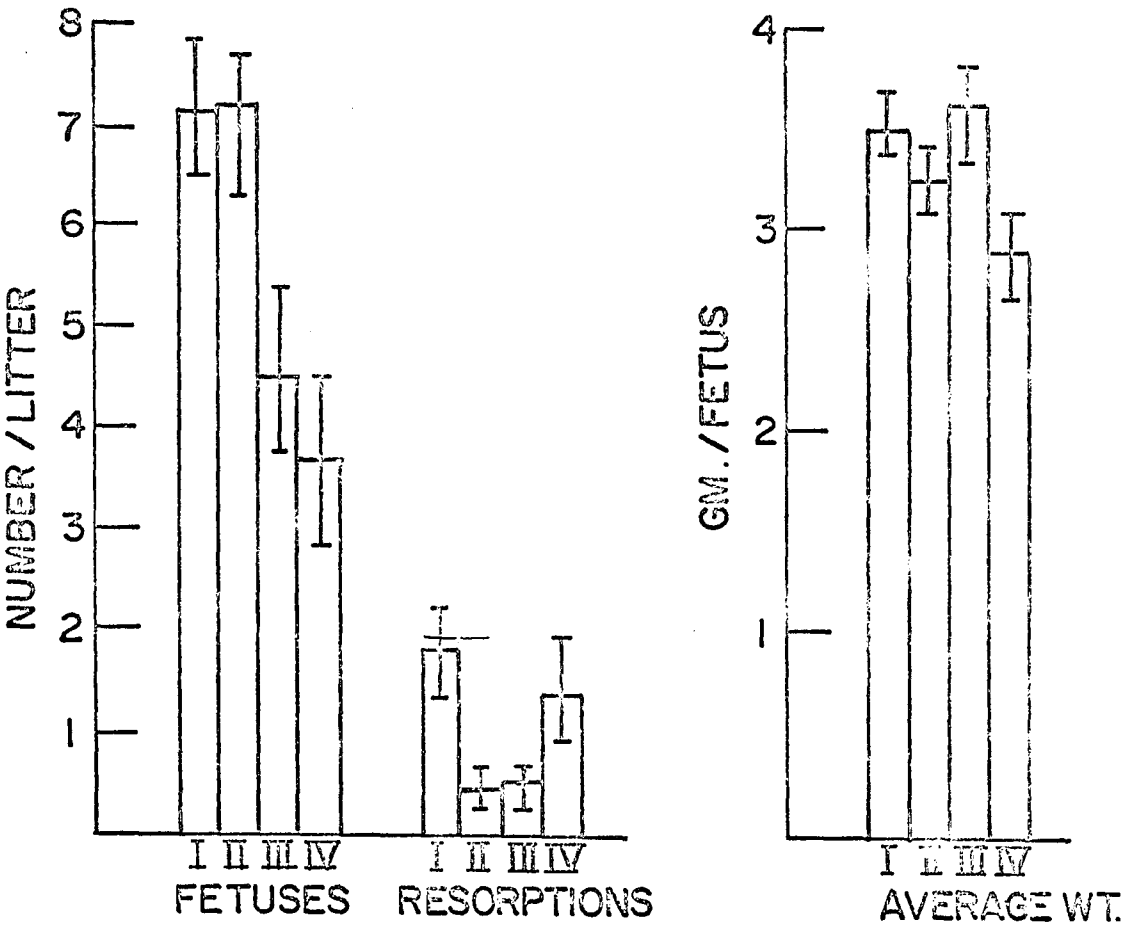


Figure 7. Number, resorptions and average weights of rat fetuses in experiment I

final body weight was the total body weight and included the weight of the litter.

There was a significant difference (5 per cent level) in the average weight of the fetuses for the different treatments. These were 3.6, 3.3, 3.7 and 2.9 gm./per fetus, respectively (Figure 7). The multiple range test (5 per cent level) indicated a significant difference between the mean fetal weight of the vitamin A-free group and the vitamin A-free plus nitrite group (III vs. IV). Gross observation indicated a more severe vitamin A deficiency in the vitamin A-free plus nitrite animals than in the vitamin A-free animals. This could result in a decreased mean fetal weight. However, liver analysis did not substantiate this in the experiment. Although Garner et al. (1958) reported that nitrite produced a vitamin A deficiency syndrome in weanling rats, yet they failed to find histologic evidence of a true vitamin A deficiency.

The vitamin A-free plus nitrite group also showed a correlation that was unique to this group. This was a significant negative correlation between adrenal gland weight and average weight of the fetuses (1 per cent level). This could be interpreted as the result of a stress response as the mean adrenal weight in this group was significantly greater than that of the vitamin A-free group (IV vs. III) and the average weight of the fetuses was significantly less.

Sinclair and Jones (1964) reported that nitrate administered to pregnant ewes resulted in smaller lambs than those

from control ewes. However, Davison et al. (1964) reported that nitrate had no effect on reproductive performance of heifers.

There was a significant difference (1 per cent level) in the mean number of resorptions for the different treatments. These were 1.8, 0.5, 0.5 and 1.5 resorption sites per treatment female (Figure 7). The multiple range test indicated that there was a significantly greater resorption rate in the group receiving laboratory ration than in the group receiving laboratory ration plus nitrite. This would not be expected. There was not a significant effect of nitrite on the resorption rate in the vitamin A-deficient groups although there was an apparent effect. Analysis of the classification of resorption durations indicated no differences in distribution between early and late resorptions in all treatment groups and is summarized in Table 4. Figure 8 is an example of a normal appearing fetus, a fetus in an early process of resorption and a late resorption site.

The location of resorption sites is given in Table 5. The ovarian pole of each respective uterine horn is the reference point and is considered as position one. Other positions are relative to this position. There were no effects of treatments on location of resorption sites regarding the right and left uterine horns. This is in agreement with Ingalls et al. (1952) who reported on the effect of anoxia in mice. They analyzed the distribution of embryos according to location in

Table 4. Classification of fetal resorptions by duration

| Group and diet | | Duration | |
|---|---|---|---|
| | | Early | Late |
| I. | Control | 18 | 16 |
| II. | Control plus nitrite | 7 | 7 |
| III. | Vitamin A-free | 4 | 5 |
| IV. | Vitamin A-free plus nitrite | 10 | 11 |

Table 5. Uterine location of resorption sites in right (R) or left (L) horn

| Group and diet | | Uterine horn | Position of resorption site | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | |
| I. | Control | R | 5 | 4 | 3 | 2 | 4 | 1 | 1 | 20 |
| | | L | 2 | 3 | 3 | 3 | 1 | 2 | | 14 |
| II. | Control | R | | 2 | 1 | 1 | 1 | | | 5 |
| | $+NO_2$ | L | 1 | 1 | 3 | 2 | 2 | | | 9 |
| III. | Vit. A-Free | R | 2 | | 1 | 1 | | | 1 | 5 |
| | | L | 2 | | 1 | 1 | | | | 4 |
| IV. | Vit. A-Free | R | 2 | | 1 | | | | 1 | 4 |
| | $+NO_2$ | L | 1 | 1 | 2 | | | 1 | | 5 |

Figure 8. Normal and abnormal products of pregnancy of a litter from vitamin A-free plus nitrite treatment. Note the normal fetus (center), fetus in process of resorption (right) and late resorption (left).

the right or left uterine horn and no finding emerged to indicate that one side provided a more favorable location for implantation and maintenance under stress than the other.

Treatments had no effect on the frequency of resorptions at any one implantation site (Table 5). Franklin and Brent (1964) reporting on the effect of uterine vascular clamping on the development of rat embryos found that there was no difference in uterine sites regarding resorption rates. However, Woollam and Millen (1961) reporting on the effects of anoxia in pregnant mice found that resorptions and stunting occurred most frequently at implantation sites at the vaginal ends of the uterine horns. However, McLaren and Michie (1960) stated that there is a significant association between congenital runting and the ovarian position in the uterine horn. A sharing of the blood supply by the ovary and the ovarian fetus was cited as a possible cause. A correlation between occupancy of ovarian position and cleft lip and palate was also cited.

Group IV, the vitamin A-free plus nitrite group, was the only treatment group that had a positive correlation (1 per cent level) between the number of resorptions and pituitary gland weights. This same group had the only significant positive correlation between adrenal weights and thyroid weights. This finding could again be interpreted as characteristic of a stress reaction imposed by treatments.

Only one fetus which was from a litter in the vitamin A-free group (group III) had lesions characteristic of the anomalies induced by maternal vitamin A deficiency. This was unusual as this fetus was from a female on the vitamin A-free diet that became pregnant on the second day breeding of the vitamin A-free diet females. Because of the lesions in the fetus from this litter, this female evidently was completely depleted of vitamin A at this time. There were eight fetuses in this litter plus one late and one early resorption. The average weight of the fetuses in this litter was 2.7 gm., whereas the mean average weight for the fetuses in the vitamin A-free diet group was 3.7 gm./fetus. The resorptions and the reduced average fetal weight in this litter would be compatible with a maternal vitamin A deficiency. The deficiency could not have been very severe or the litter would not have been carried to term (Warkany and Schraffenberger, 1946). The ocular congenital anomalies induced as a result of maternal vitamin A deficiency will be discussed in experiment II under the topic of fetal analyses.

Tables 16 and 17 of mean values for experiment I with standard errors of the means are found in the appendix.

## MATERIALS AND METHODS-EXPERIMENT II

Only deviations from experiment I will be described for experiment II. Unless otherwise stated, materials and methods in experiment II were the same as experiment I.

### Experimental Design

#### Animals

Disease-free rats[1] were purchased to provide a source of rats for use on experiment II. The animals were handled as in experiment I. The animals were quartered in an air conditioned room in experiment II.

#### Division of groups

Eight treatment groups were established on the basis of the female and male diets as follows:

I. Control diet (female) X control diet (male)

II. Control diet (female) X control diet plus nitrite (male)

III. Control diet plus nitrite (female) X control diet (male)

IV. Control diet plus nitrite (female) X control diet plus nitrite (male)

V. Vitamin A-free diet (female) X control diet (male)

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[1]Charles River Breeding Laboratories, Inc., North Wilmington, Massachusetts.

VI. Vitamin A-free diet (female) X control diet plus nitrite (male)

VII. Vitamin A-free diet plus nitrite (female) X control diet (male)

VIII. Vitamin A-free diet plus nitrite (female) X control diet plus nitrite (male)

Female rats in the control diet groups, control diet plus nitrite groups and vitamin A-free diet groups were distributed to these groups at 30 days of age. Ultimate distribution to groups was made at the time of determination of day zero of pregnancy. Treatment of the servicing males determined the ultimate distribution to groups.

Unequal group sizes were used with a total of 99 animals. Group sizes were as follows: group 1, 12; group II, 11; group III, 7; group IV, 15; group V, 9; group VI, 8; group VII, 17; and group VIII, 20.

Method of handling

Female rats in the control diet plus nitrite groups as well as the nitrite males received 0.25 per cent $KNO_2$ (415 p.p.m. nitrite nitrogen) in the drinking water from 30 days of age until the termination of the experiment. Drinking waters were freshly prepared every day by dissolving and dispensing the required amount of potassium nitrite in the final volume of water in watering bottles. All nitrite females in these

groups and nitrite males received nitrite for not less than 60 days before breeding.

Nitrite was administered in the water in experiment II rather than in the feed as in experiment I because of ease in insuring a more uniform mixing and because there should be less loss of nitrite by reduction in watering bottles than in the feed during the 24-hour periods between feedings and waterings. In experiment I the nitrite was added to the feed rather than the water because the animals in experiment I were housed in facilities that were not temperature controlled and became very hot at times. Water consumption increased at these times and was more variable than feed consumption. Animals in experiment II were housed in air conditioned facilities and water consumption was not variable with outdoor temperatures. To insure the presence of the added nitrite in the water, one analysis was made of treatment waters prepared 24 hours earlier from potassium nitrite handled in a manner like that during the experimental period. An adaptation of the colorimetric alphanaphthylamine method was employed (American Public Health Association, 1938).

Breeding was conducted in the same manner as in experiment I. When nitrite males were placed overnight in cages with non-nitrite females, non-nitrite water was available. When non-nitrite males were placed overnight with nitrite females, again non-nitrite water was available during the mating period.

Rats receiving the control diet were fed a commercial laboratory ration that is considered to be free of salmonella organisms and would be indicated for use with disease-free rats.[1]

The purified vitamin A-free diet was the same as that used in experiment I and was fed from 30 days of age to the end of the experiment. Forty I.U. of vitamin A were administered orally every ten days until day zero of pregnancy.

## Body Weight Analysis

Only final body weights were recorded at day 20 of pregnancy.

## Blood Analyses

Blood analyses were performed in the same manner as in experiment I. Pre-breeding hemoglobin, methemoglobin and PCV were determined on those females that were on control ration and control ration plus nitrite from 30 days of age. Day 20 hemoglobin, methemoglobin and PCV were determined on all females.

## Liver Analyses

Liver vitamin A analysis was initially performed on pooled liver samples. The pooling criteria were the dietary

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[1]Rockland Mouse/Rat Diet. Teklad, Inc., Monmouth, Illinois.

treatment regimen of the females thus making four pooled samples. A later analysis was performed on the vitamin A-free diet and vitamin A-free diet plus nitrite groups by pairing samples from each group as to the length of time that the animals were on the vitamin A-free diet.

## RESULTS AND DISCUSSION - EXPERIMENT II

Discussion that is common for both experiments I and II will be found in experiment I.

### Body Weight Analysis

The mean final body weights were 341, 356, 338, 328, 338, 322, 321 and 315 gms., respectively (Figure 9). There were no significant differences among the means of these groups.

In experiment I, nitrite was given in the feed which reduced palatability and encouraged feed wastage. Although paired feeding was used the amount of feed wasted could not be determined. There were significant differences among mean final body weights of the treatment groups. However, in experiment II when nitrite was in the water rather than the feed there was no significant treatment difference in mean final body weights. This would indicate that nitrite ingestion *per se* was without effect on rate of growth.

### Blood Analyses

#### Pre-breeding

The pre-breeding PCV averaged 39.5, 38.3, 44.5 and 44.8 per cent for group I, control diet (female) X control diet (male); group II, control diet (female) X control diet plus nitrite (male); group III, control diet plus nitrite (female) X control diet (male); and group IV, control diet plus nitrite

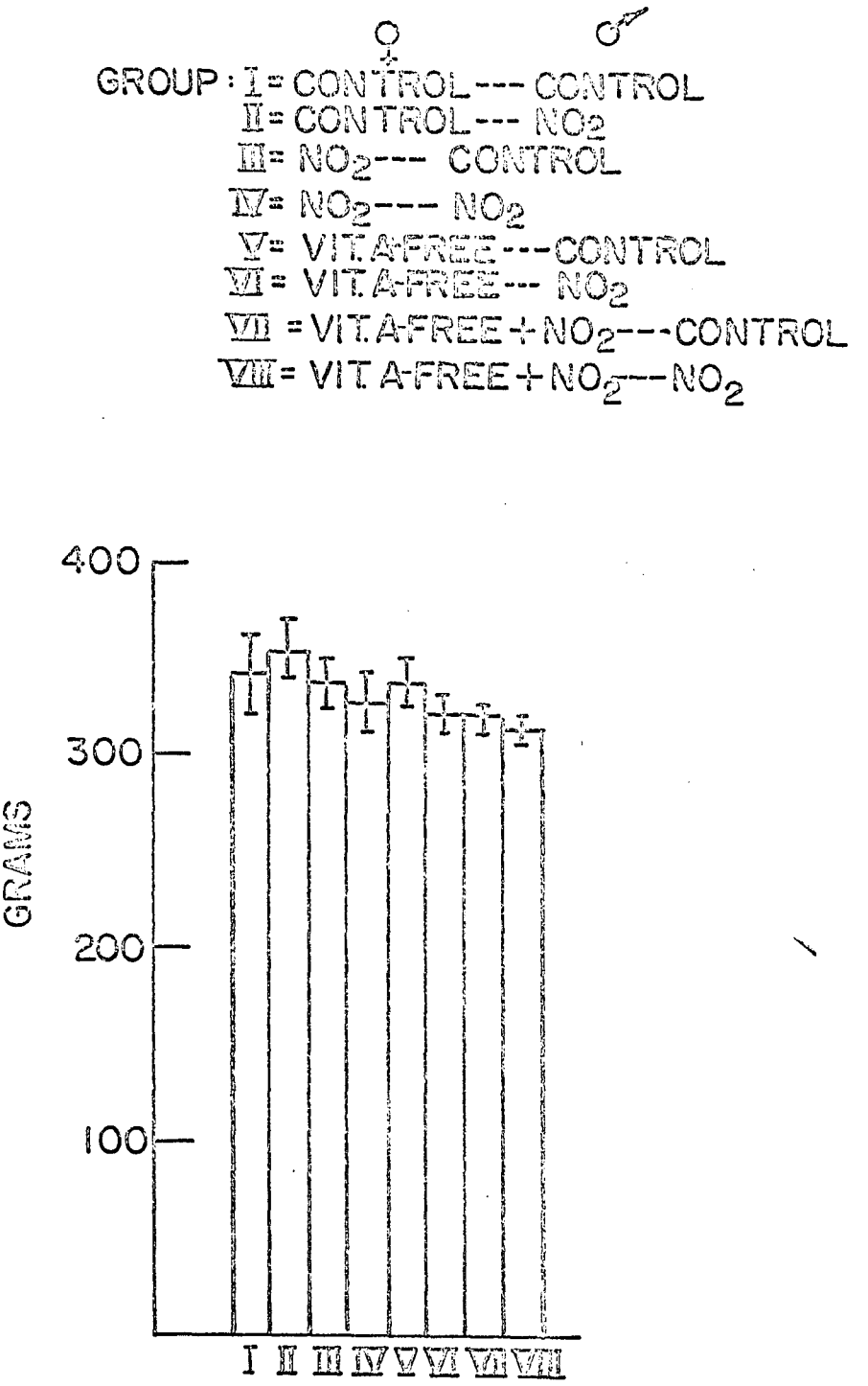



Figure 9. Final body weights of rats in experiment II

(female) X control diet plus nitrite (male), respectively (Figure 10). Treatment differences were significant at the 1 per cent level. The multiple range test (5 per cent level) indicated that there were a significant increases in the means of the PCV of the groups receiving nitrite from those not receiving nitrite (III and IV vs. I and II). The animals receiving nitrite had been on nitrite-containing water from 30 days of age and had received nitrite for no less than 60 days.

The pre-breeding mean hemoglobin values were 15.5, 15.2, 17.0 and 16.5 gm./100 ml. of blood, respectively (Figure 11). The treatment effect differences were significant at the 1 per cent level. There was a significant difference (5 per cent level) between group II which did not receive nitrite and groups III and IV which did receive nitrite, and also between group I (no nitrite) and group III (nitrite).

Pre-breeding mean methemoglobin values were 0.6, 0.3, 1.4 and 1.0 gm./100 ml. of blood, respectively (Figure 12). Treatment effect differences were significant at the 5 per cent level and groups receiving nitrite had significantly (5 per cent) higher methemoglobin values than did groups not receiving nitrite (III and IV vs. I and II).

It was evident in the pre-breeding hematologic analyses that the ingestion of nitrite caused an increase in methemoglobin production above control values, although the amount of methemoglobin detected was not very great. Increased

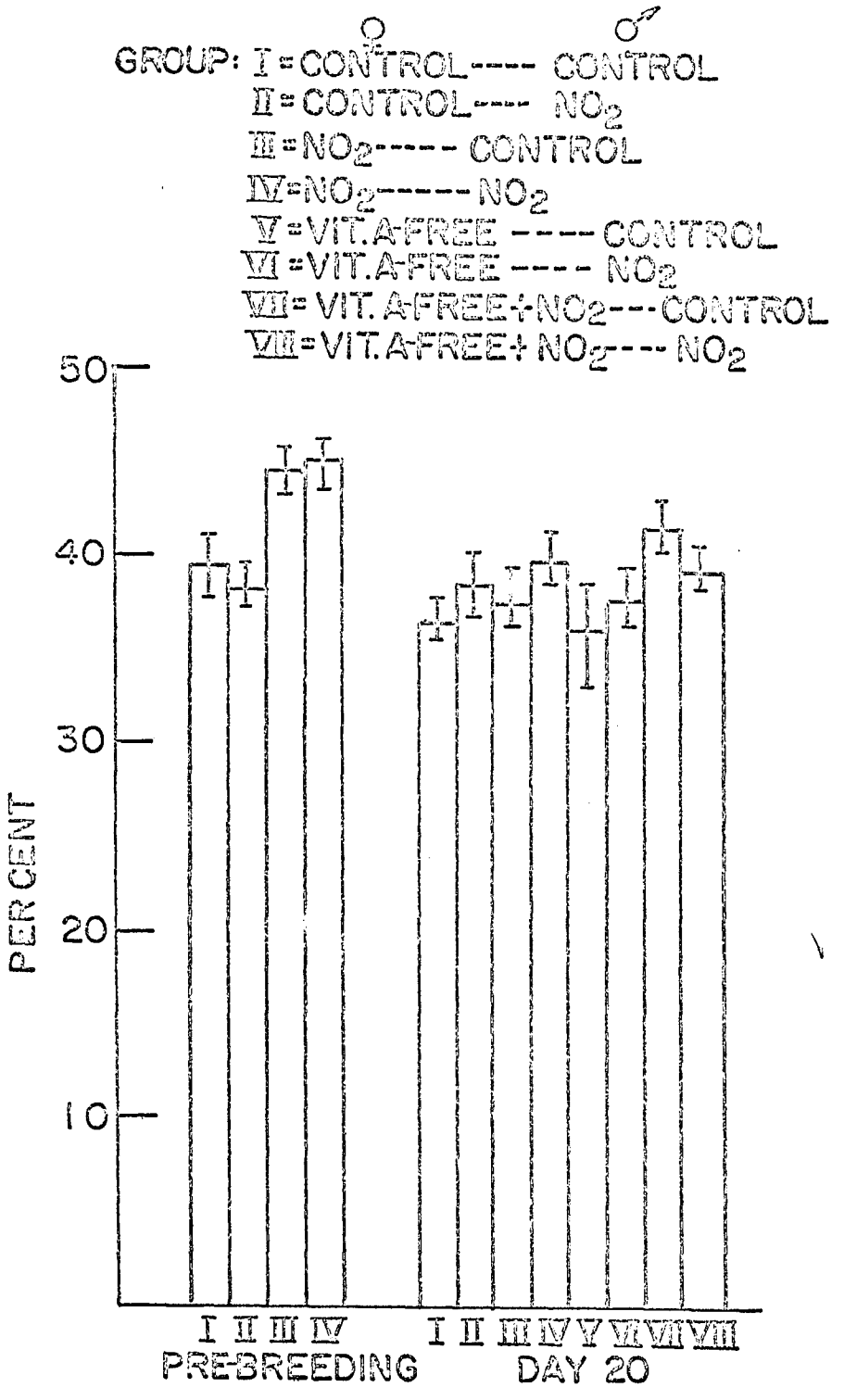



Figure 10. Pre-breeding and day 20 packed cell volumes (PCV) of rats in experiment II

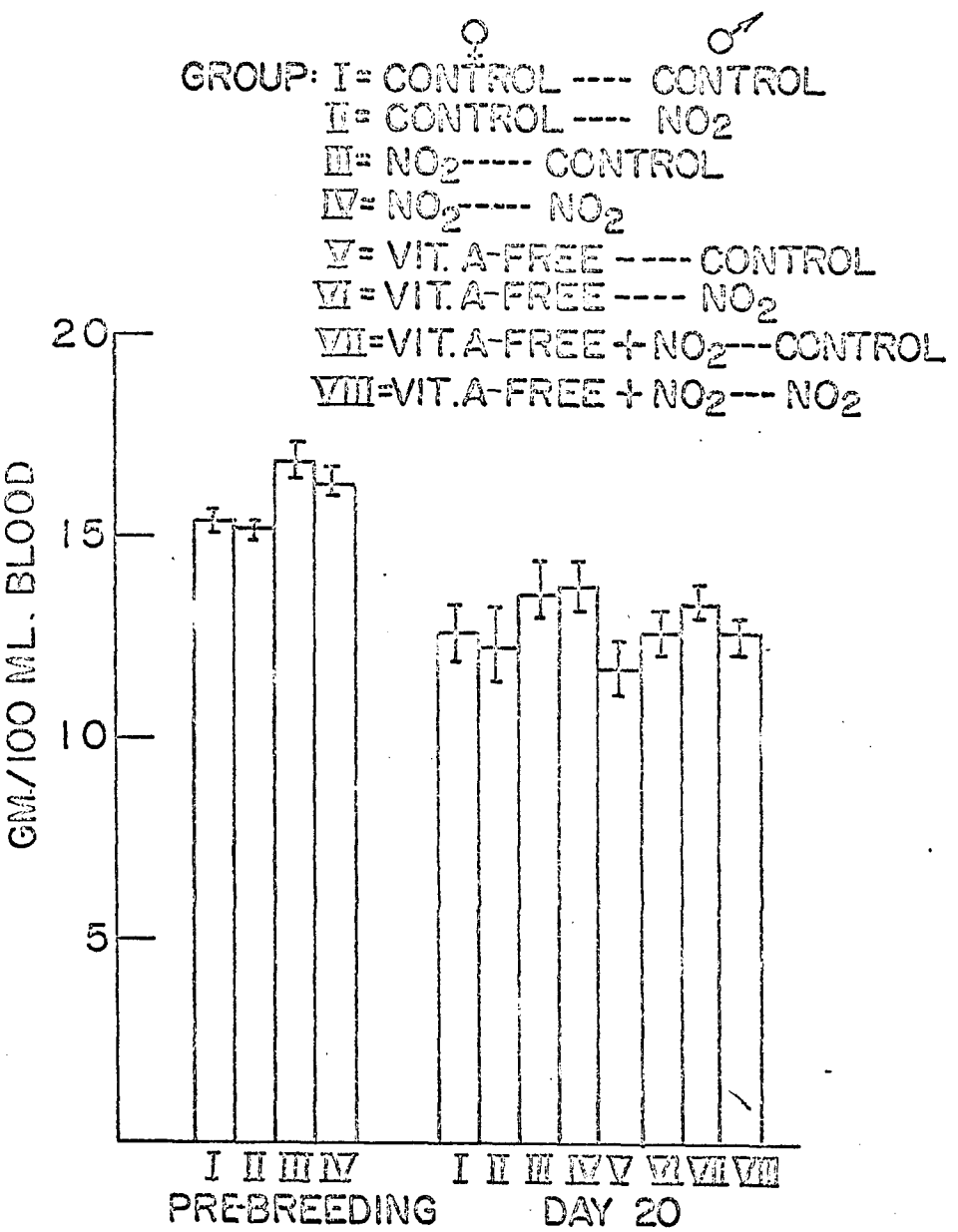



Figure 11. Pre-breeding and day 20 hemoglobin values of rats in experiment II

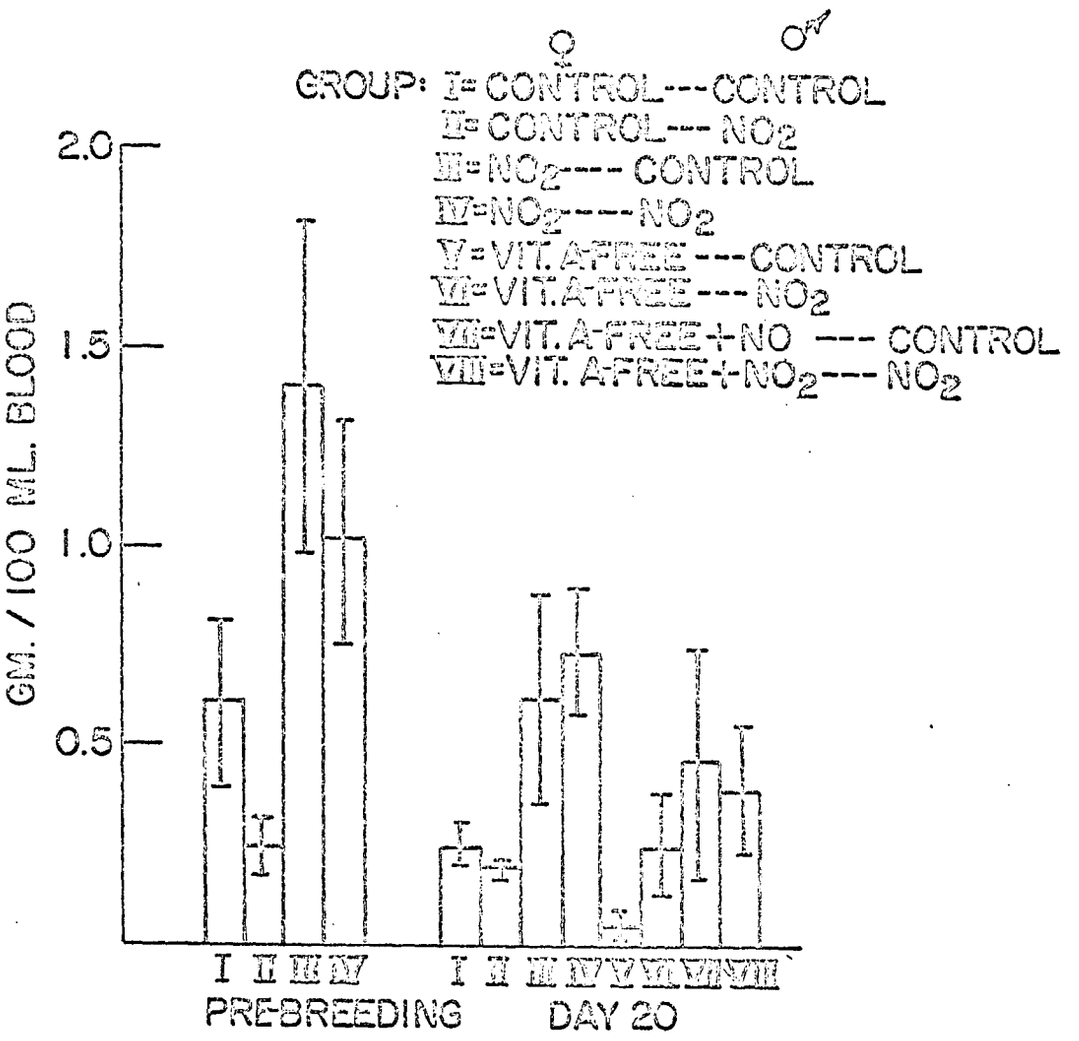



Figure 12. Pre-breeding and day 20 methemoglobin values of rats in experiment II

hemoglobin and PCV were evident in the nitrite groups (III and IV). It would seem that although the amount of methemoglobin was not great in the nitrite groups, there must have resulted a degree of anoxia that was sufficient to stimulate erythropoietic activity as evidenced by increased hemoglobin and PCV in these groups. All values would be considered to be in a normal range.

A positive correlation at the 5 and 1 per cent levels existed only in the nitrite groups (III and IV, respectively) between pre-breeding hemoglobin and pre-breeding PCV. A positive correlation between methemoglobin and these variables would have been expected as increased methemoglobin would result in an anoxic stimulus with a resultant increased erythropoieses, but a positive correlation was not evident upon examination of the data.

Day 20

The mean day 20 PCV values were 36.6, 38.5, 37.8, 40.0, 36.0, 37.9, 41.7 and 39.2 per cent for groups I through VIII, respectively (Figure 10). There was no significant difference among treatment groups. Values were considered normal. There also was no significant difference among means of the day 20 hemoglobin values. These values were 12.7, 12.4, 13.8, 13.9, 11.9, 12.7, 13.4 and 12.7 gm./100 ml. of blood, respectively (Figure 11).

The mean day 20 methemoglobin values were 0.26, 0.20, 0.62, 0.74, 0.05, 0.26, 0.47 and 0.39 gm./100 ml. of blood,

respectively (Figure 12). There was no significant difference among means.

The day 20 blood analyses did not show any significant effects of treatments. With the exception of the PCV of group III and of the hemoglobin of group VIII, all groups receiving dietary nitrite had higher apparent values than the corresponding groups not receiving nitrite (Figures 10, 11). A positive correlation across all treatments at the 5 per cent level was found between day 20 methemoglobin and both day 20 hemoglobin and day 20 PCV. A positive correlation between day 20 hemoglobin and PCV was also present, as would be expected. The day 20 methemoglobin values in the groups receiving nitrite were lower than the pre-breeding values in the corresponding groups. The day 20 methemoglobin values in groups receiving nitrite were also lower than the corresponding groups in experiment I. The lower methemoglobin at day 20 was not expected. Rats apparently adapt rapidly to the constant exposure to methemoglobin-producing substances by increased rate of reduction of methemoglobin. During the course of these experiments reported here, a separate group of rats was administered drinking water containing 0.25 per cent potassium nitrite (415 p.p.m. nitrite nitrogen). Methemoglobin determinations were made on the third day of receiving the nitrite in the drinking water. The mean methemoglobin value was 3.5 gm./100 ml. of blood. Rats in experiment II that received the same level of nitrite in the water for at

least 60 days had mean methemoglobin levels of 1.2 gm./100 ml. of blood. Day 20 values, which were obtained from 30 to 60 days later, are given above.

## Small Organ Weights Analyses

Day 20 mean adrenal gland weights were 17.8, 17.1, 22.2, 18.6, 14.9, 13.0, 15.8 and 15.4 mg./100 gm. of body weight, respectively (Figure 13). Treatment differences were significant at the 1 per cent level. The multiple range test indicated that group III had a significantly greater mean adrenal weight than all other groups except groups I and IV (1 per cent level). The diets of groups III and IV were the control diets plus nitrite and the results were as expected, although there was an apparent difference in the mean adrenal weights between these two groups. The diets of groups I and II were the control diet. There was a difference between adrenal weights on the control diet and control diet plus nitrite, i.e., group II and group III (5 per cent level). Nitrite ingestion in experiment I also produced a significant increase in adrenal weights over the same groups without nitrite. The increased adrenal weights are apparent in the vitamin A-free plus nitrite groups compared with the vitamin A-free groups, i.e., groups V and VI vs. groups VII and VIII, respectively.

Day 20 mean thyroid gland weights were 5.1, 4.0, 4.6, 4.9, 4.4, 6.2, 5.0 and 5.4 mg./100 gm. of body weight, respectively

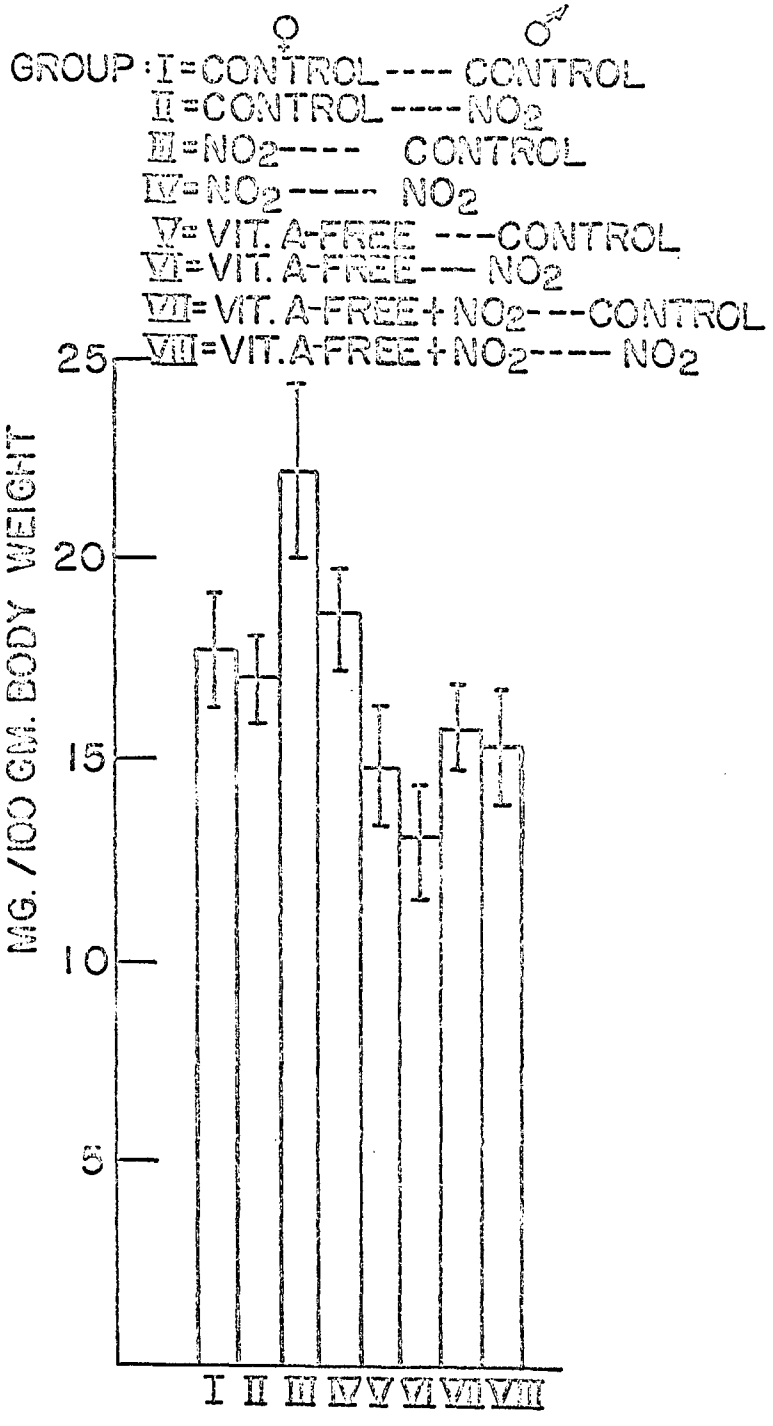



Figure 13. Adrenal weights of rats in experiment II

(Figure 14). Treatment differences were not significant nor was there any apparent effect of nitrite on thyroid weight.

Day 20 mean pituitary gland weights were 2.7, 2.3, 2.7, 3.0, 2.5, 2.8, 2.5 and 2.5 mg./100 gm. of body weight, respectively (Figure 14).

Treatment differences were not significant nor was there any apparent effect of nitrite on pituitary weight.

There was a positive correlation at the 5 per cent level of significance between pituitary weight and the adrenal weight only in groups VII and VIII. Both of these groups were on the vitamin A-free plus nitrite ration. This would be indicative of an expected stress response.

There was a positive correlation between pituitary weight and thyroid weight (5 per cent level) only in groups III and VII. The dietary regimen of these groups was control plus nitrite and vitamin A-free plus nitrite, respectively. This finding could be interpreted as indicative of an effect of nitrite on thyroid function, but other groups that received nitrite did not indicate a significant correlation of this type. Thus, this interpretation may not be valid.

A positive correlation (5 per cent level) between thyroid weight and adrenal weight existed only in group V, a vitamin A-free group. This could be indicative of a stress response.

An interesting negative correlation (5 per cent level) between adrenal weight and both day 20 hemoglobin and PCV was found in group VIII which was on a vitamin A-free plus nitrite

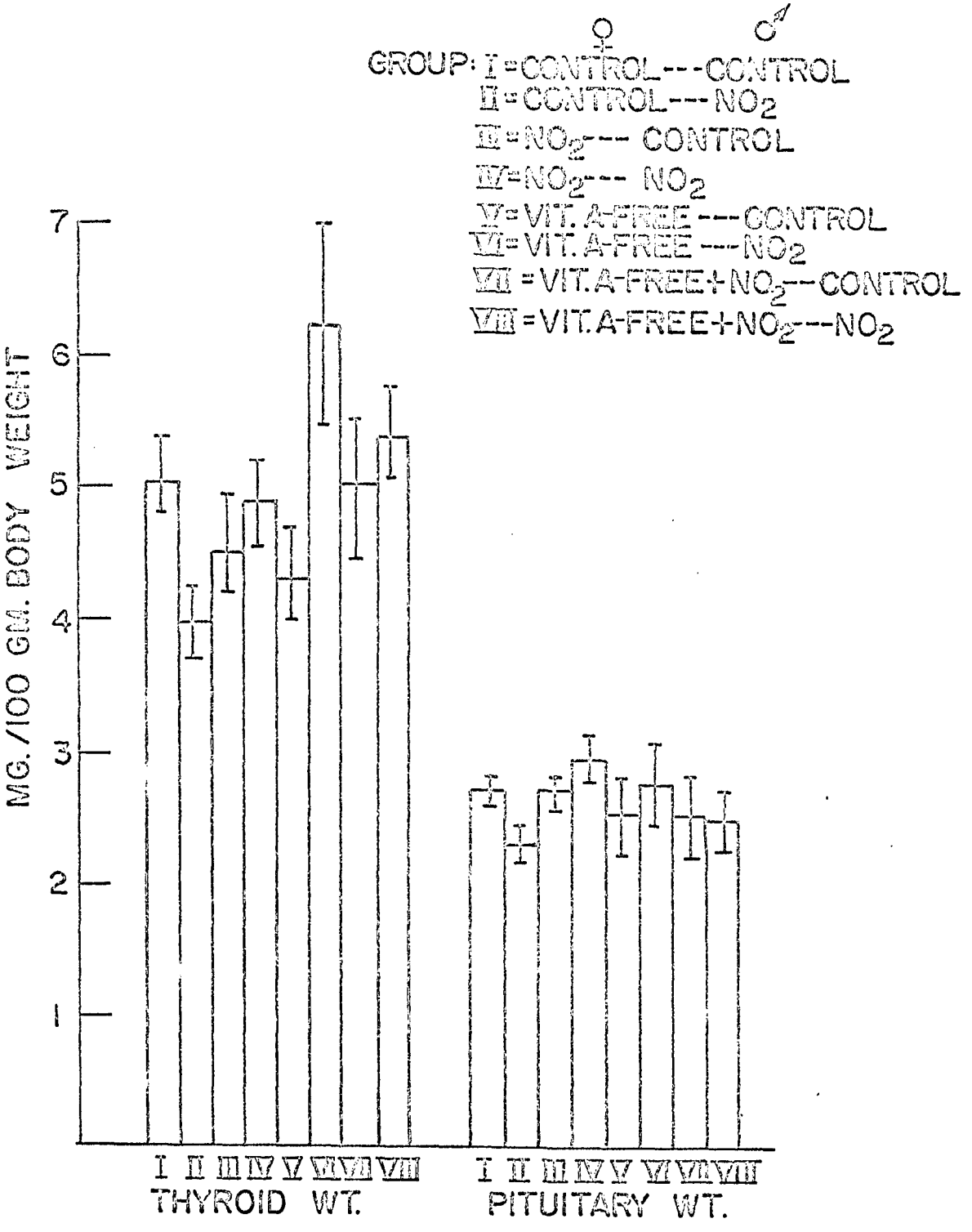



Figure 14. Thyroid and pituitary weights of rats in experiment II

diet. A negative correlation between adrenal weight and day 20 PCV was evident in group I which was on the control diet and group IV which was on the control diet plus nitrite. If group I would not have shown this correlation, the following explanation would seem valid. The animals were on nitrite-containing rations and the nitrite produced a sufficient adrenal stress reaction which resulted in a retention of fluid and a resulting hemodilution that was evident on day 20. Day 20 blood values ordinarily showed a decrease in values compared with earlier values because of pregnancy.

## Liver Analyses

Day 20 mean liver weights were 3.5, 3.4, 3.3, 3.7, 3.4, 2.8, 3.2 and 3.2 gm./100 gm. of body weight, respectively (Figure 15). The differences were significant at the 5 per cent level. The multiple range test indicated that dietary nitrite did not have any effect on reducing liver weight when comparisons were made between groups receiving the same dietary regimen with the exception of nitrite. These were the same findings as in experiment I, where this was discussed.

Potassium nitrite in the drinking water reduced liver vitamin A in the control plus nitrite sample compared with the sample from groups receiving the control ration (Table 6). Nitrite administered to the vitamin A-free groups did not reduce liver vitamin A when compared with the vitamin A-free groups not receiving nitrite (Table 6). Values for both of

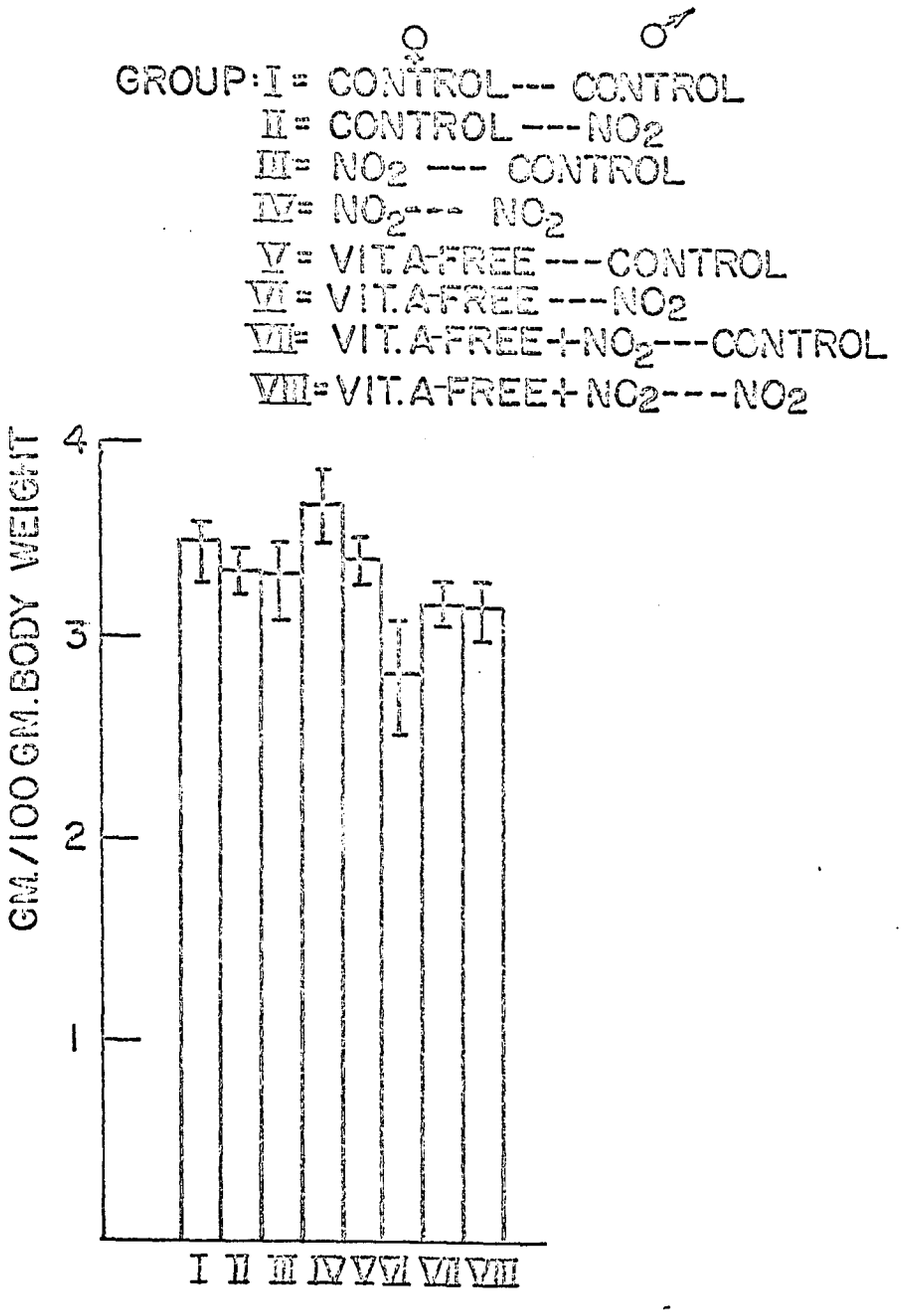



Figure 15. Liver weights of rats in experiment II

Table 6. Liver vitamin A analysis

| Diet | Groups | Vitamin A (mcgm./gm. of liver) |
|---|---|---|
| Control | I, II | 71.6 |
| Control+$NO_2$ | III, IV | 61.0 |
| Vitamin A-free | V, VI | 2.8 |
| Vitamin A-free+$NO_2$ | VII, VIII | 14.2 |
| Vitamin A-free (paired) | V, VI | 5.0 |
| Vitamin A-free+$NO_2$ (paired) | VII, VIII | 22.6 |

the vitamin A-free groups were low. These were the same results as obtained in experiment I.

It was initially hypothesized that the results of the vitamin A analysis involving the vitamin A-free groups could be due to the fact that the animals in these groups were on the vitamin A-free ration longer than those in the vitamin A-free plus nitrite groups. Thus, they might have been more depleted of vitamin A at day 20. To eliminate any effect of this nature a second group of samples was formed from these groups by pairing animals as to the number of days from weaning to termination of the experiment. This analysis was performed at a later time from the first analyses. The results are indicated in Table 6 as "paired". The pairing had an effect on absolute values of vitamin A but did not alter the relationship. The sample from vitamin A-free plus nitrite groups had higher liver vitamin A than the sample from groups

not receiving nitrite. Discussion of this is found in experiment I. It is of interest to note the great differences in the pooled liver vitamin-A values of the control diet groups from experiments I and II (327.15 vs. 71.6 mcgm./gm. liver, respectively) (Tables 2, 6). The two groups received different brands of commercial laboratory animal rations. It seems that there must be differences in the vitamin A contents of the two rations if liver vitamin A stores are an indication.

## Fetal Analyses

All the females in which sperm were found in the vaginal smear did not become pregnant, nor did all those that became pregnant have viable fetuses at day 20. The data are summarized in Table 7. Chi-square analysis indicated no significant differences due to treatments in the pregnancy rate, number of females delivering live fetuses without resorptions, number of females with one resorption and number with more than one resorption but less than 100 per cent resorptions. The only treatment groups with females exhibiting 100 per cent resorptions of litters were the vitamin A-free plus nitrite females (VII and VIII).

The mean number of live fetuses per female was 8.9, 7.9, 10.6, 8.7, 9.2, 8.5, 8.4 and 7.0, respectively (Figure 16). The treatment differences were not significant. There was no effect of maternal nitrite ingestion on the number of fetuses in the groups receiving the control ration (III and IV).

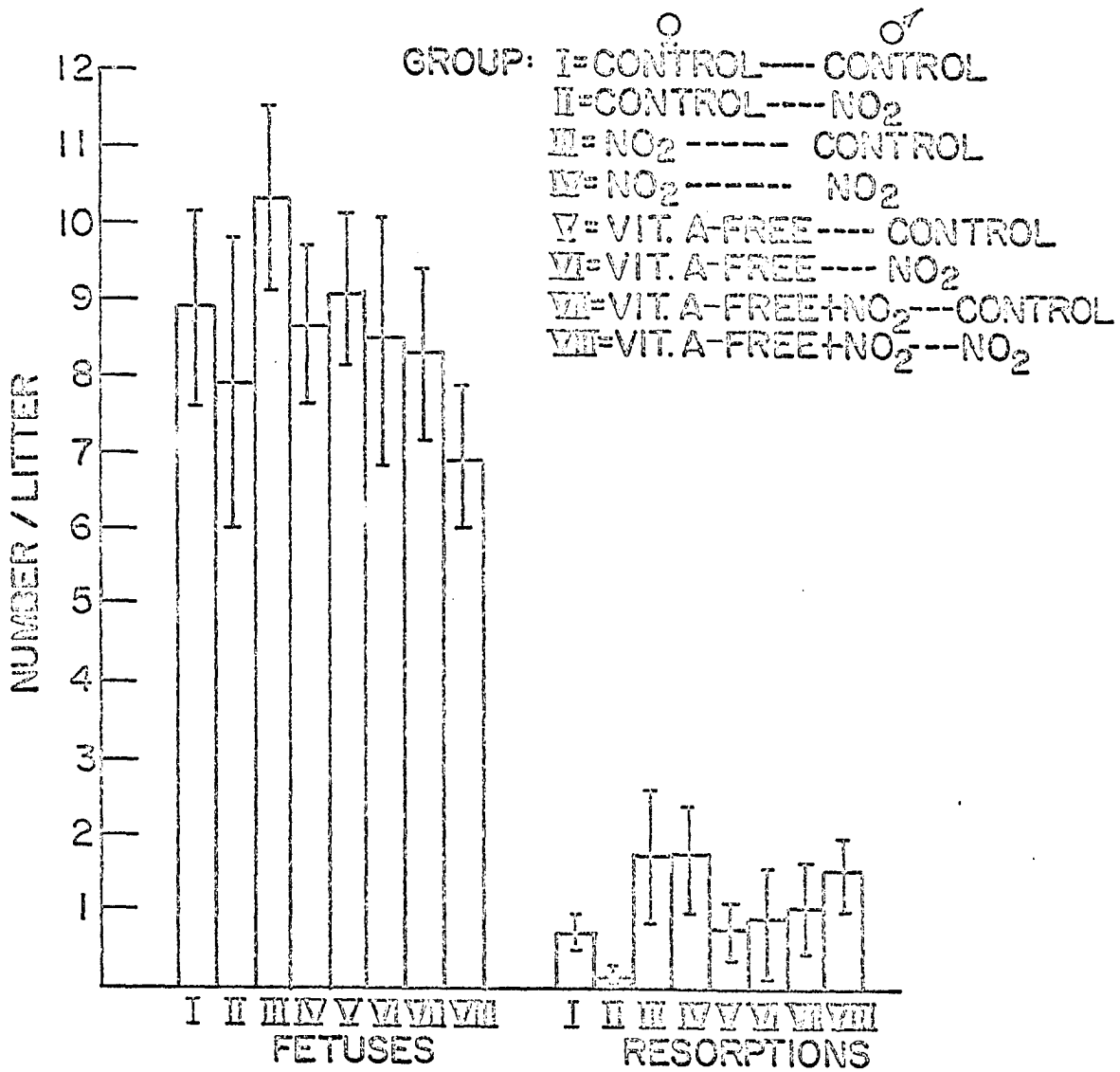



Figure 16. Number and resorptions of rat fetuses in experiment II

Table 7. Reproductive performance of rats in the eight treatment groups

| | Group | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII |
| Mated females | 12 | 11 | 7 | 15 | 9 | 8 | 17 | 20 |
| No. with implantations | 10 | 6 | 7 | 14 | 9 | 7 | 15 | 20 |
| Litters | | | | | | | | |
| all alive | 3 | 4 | 3 | 6 | 5 | 5 | 9 | 4 |
| 1 resorption | 6 | 2 | 2 | 3 | 2 | 1 | 3 | 8 |
| >1 resorption <100% | 1 | 0 | 2 | 5 | 2 | 1 | 2 | 6 |
| 100% resorptions | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 |

There was an apparent effect of nitrite ingestion on decreasing the number of fetuses in the groups receiving the vitamin A-free ration (VII and VIII).

The male had an apparent effect upon the mean number of fetuses. The mean number of fetuses in all groups with a nitrite male was less than that in groups on the same dietary regimen with a control male (II, IV, VI, VIII vs. I, III, V, VII). This was the only apparent effect of treatment of the male upon pregnancy in the rat in this experiment. A comparison was made between the mean number of fetuses resulting from control males vs. the mean number of fetuses resulting from nitrite-treated males. There was no significant difference.

There has been little work in experimental teratogenesis involving the effect of agents on the male and subsequent

congenital malformations in offspring. Lukwak-Mann (1964) described effects produced as a result of thalidomide treatment of male rabbits. Reduction in littering and congenital malformations were produced. It thus is reasonable to hypothesize that other agents mediated through the male could have comparable effects.

There was no significant effect of treatments on the mean fetal weights. These were 3.9, 3.7, 3.7, 3.6, 3.4, 3.5, 3.4 and 3.5 gm./per fetus, respectively (Figure 17).

There was no significant effect of treatments on the mean number of resorptions. These were 0.8, 0.2, 1.7, 1.7, 0.8, 0.9, 1.1 and 1.6 resorption sites per treatment female for the respective groups (Figure 16). There were no statistically significant effects of treatments on the distribution of duration of resorptions in the various groups, although group VIII had a greatly increased number of late resorptions compared with early resorptions. The classification of the duration of resorptions is given in Table 8. Analysis of the classification of resorption durations indicated no differences in distribution between early and late resorptions in all treatment groups although there is an apparent effect in group VIII, which is the contribution made by the females that had litters exhibiting congenital ocular anomalies.

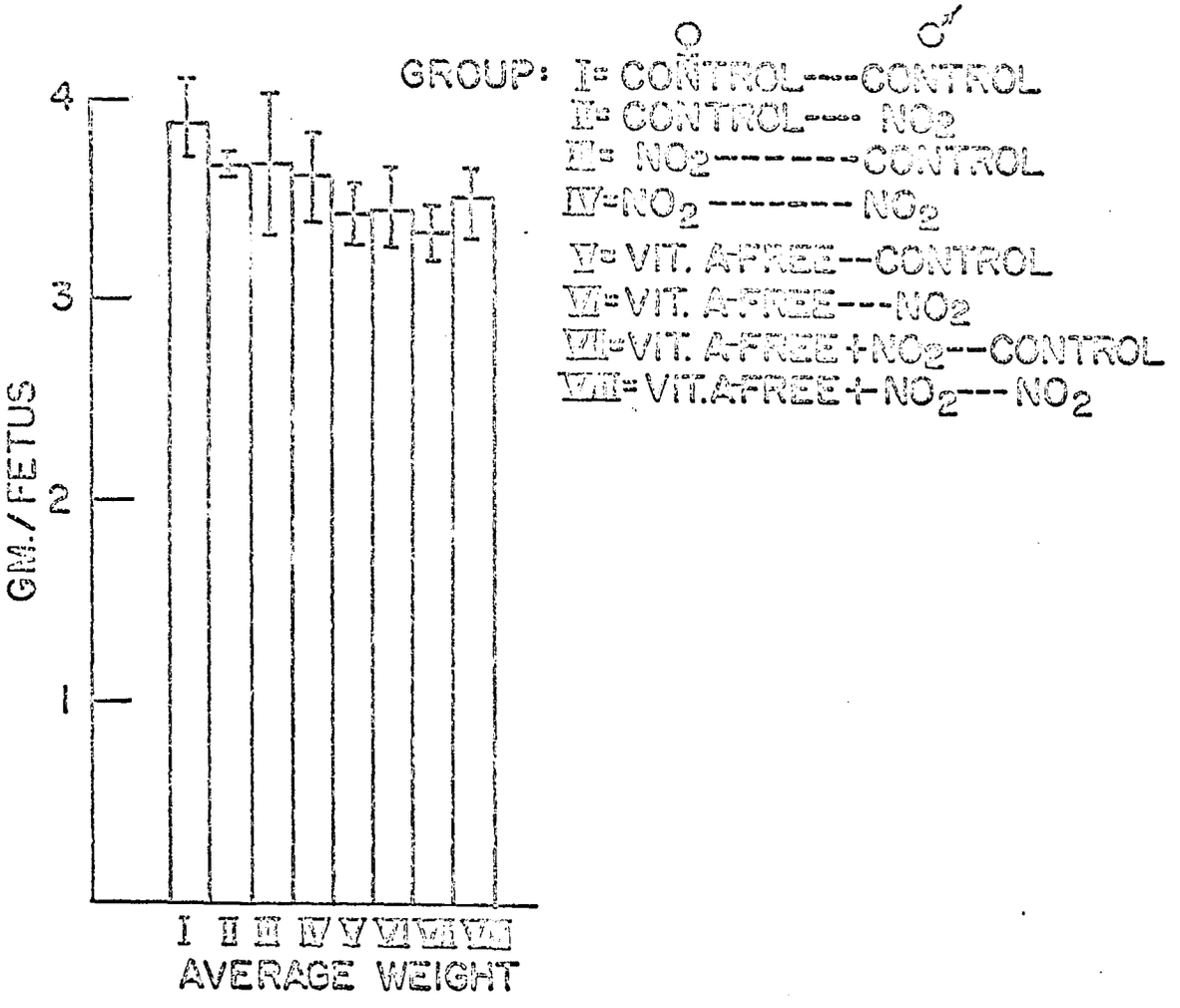



Figure 17. Average weights of rat fetuses in experiment II

Table 8. Classification of fetal resorptions by duration

| Group and diet | | | Duration | |
|---|---|---|---|---|
| | | | Early | Late |
| | Female | Male | | |
| I | Control | Control | 2 | 7 |
| II | Control | $NO_2$ | 0 | 2 |
| III | $NO_2$ | Control | 8 | 4 |
| IV | $NO_2$ | $NO_2$ | 17 | 9 |
| V | Vit. A-free | Control | 6 | 1 |
| VI | Vit. A-free | $NO_2$ | 6 | 1 |
| VII | Vit. A-free+$NO_2$ | Control | 3 | 6 |
| VIII | Vit. A-free+$NO_2$ | $NO_2$ | 6 | 21 |

The location of resorption sites is given in Table 9. The ovarian pole of each respective uterine horn is the reference point and positions are numbered from that end toward the cervices. There was no effect of treatments on location of resorption sites regarding right and left uterine horns.

Gross examinations of the fetuses did not reveal any abnormalities. Warkany and Schraffenberger (1946) reporting on malformations induced in rats by maternal vitamin A deficiency described an external abnormality of the eye which was called open eye and was characterized by a red discoloration between the rudimentary lids.

Table 9. Uterine location of resorption sites in right (R) or left (L) horn

| Group | and diet | Uterine horn | Position of resorption sites | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
| I | F[a] Control, | R | 1 | | 2 | 1 | | | | | 4 |
| | M Control | L | | 1 | | 1 | 1 | 2 | | | 5 |
| II | F Control, | R | | | | | | | | | 0 |
| | M $NO_2$ | L | 1 | | | 1 | | | | | 2 |
| III | F $NO_2$, | R | 1 | 2 | | | | 1 | 2 | | 6 |
| | M Control | L | 2 | | 1 | 1 | 2 | | | | 6 |
| IV | F $NO_2$, | R | 3 | 2 | 4 | 2 | 2 | 1 | | | 14 |
| | M $NO_2$ | L | 2 | 2 | 3 | 1 | 3 | 1 | | | 12 |
| V | F VAF[b], | R | 1 | 1 | 1 | 1 | 1 | | | | 5 |
| | M Control | L | | | 1 | 1 | | | | | 2 |
| VI | F VAF, | R | 1 | 1 | 1 | 1 | 1 | | | | 5 |
| | M $NO_2$ | L | | | 2 | | | | | | 2 |
| VII | F VAF+$NO_2$, | R | 1 | | 1 | 1 | | | | | 3 |
| | M Control | L | 2 | | 1 | | | 1 | 1 | 1 | 6 |
| VIII | F VAF+$NO_2$, | R | 2 | 3 | 3 | 2 | 1 | | | | 11 |
| | M $NO_2$ | L | 4 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 16 |

[a] F = female, M = male.

[b] VAF = vitamin A-free.

Serial sections were made of the head region (primarily through the eyes) of one fetus from each litter. Wilson and Warkany (1950) reporting on malformations induced by vitamin A deficiency stated that they had never found any other anomaly existing in the absence of ocular malformation.

Warkany and Schraffenberger (1946) describing serial sections of eyes of fetuses from vitamin A deficient female rats found that of 32 eyes examined in great detail all 32 showed a fibrous retrolenticular membrane. Other abnormalities were found at only a slightly lesser frequency. Because the fibrous retrolenticular membrane is the most common characteristic congenital abnormality of vitamin A deficiency, it was the criterion used for detecting ocular malformations and was the most frequently occurring abnormality. Figure 18 is a section of a normal eye of a day 20 rat fetus through the optic stalk and can be used for comparison with the sections depicting abnormalities. Representative sections of abnormalities will be described in the following paragraphs.

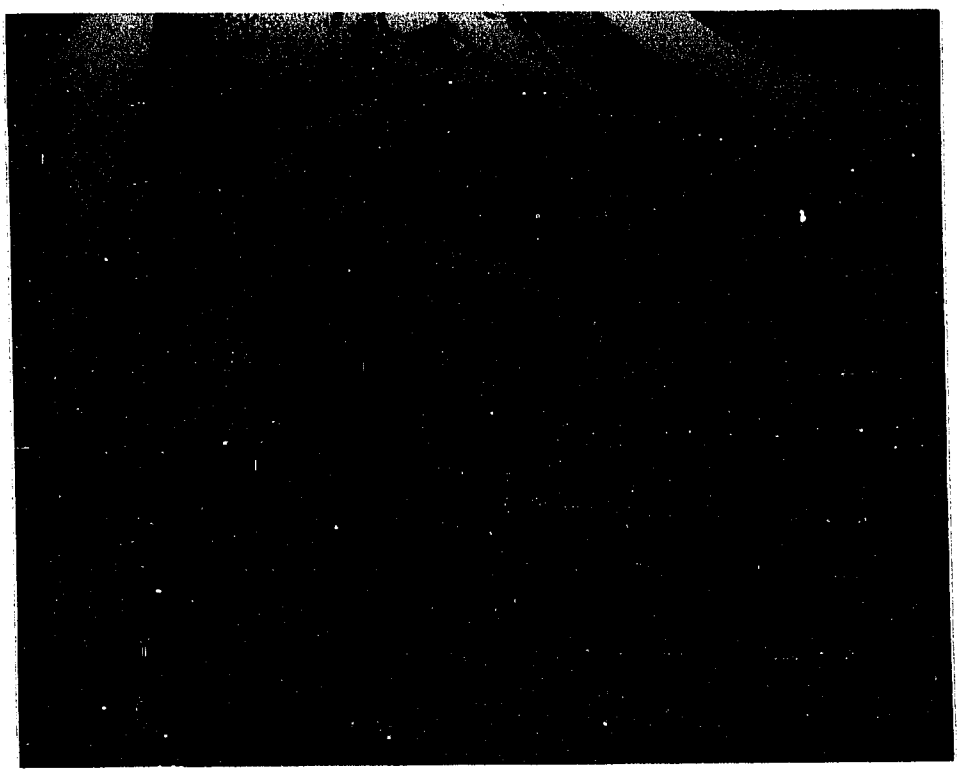
The vitreous space is occupied in varying degrees by a dense network of fibrous tissue within which capillaries and branches of hyaloid vessels may be discerned (Figures 19, 20). This retrolenticular tissue is definitely overgrown as compared with the retrolenticular tissue in normal fetuses. The fibrous connective tissue which is usually most dense immediately behind the lens decreases in thickness toward the equator of the lens, where it ends (Figure 21). The internal leaf of the retina may form several folds and may be distinctly everted behind the point where the retrolenticular and extraocular mesoderm join at a retinal cleft (Figures 19, 20). This retinal cleft with retinal eversion is near the optic nerve. The center of the retinal cleft is transversed by a thick

Figure 18. Section of the eye of a normal day 20 rat fetus. H. and E. Approximately 40X.

L indicates eyelid; CS, conjunctival space; Co, cornea; AC, anterior chamber; I, iris; Le, lens; IR, internal leaf of retina; V, vitreous; OV, space of optic vesicle; ER, external leaf of retina; ON, optic nerve.

Figure 19. Section of the left eye of an abnormal day 20 rat fetus. H. and E. Approximately 45X.

Female No. 102v. Group VIII, vitamin A-free plus nitrite diet. RM indicates fibrous retrolenticular membrane connected with extraocular mesoderm; Col, coloboma; E, eversion of retina.



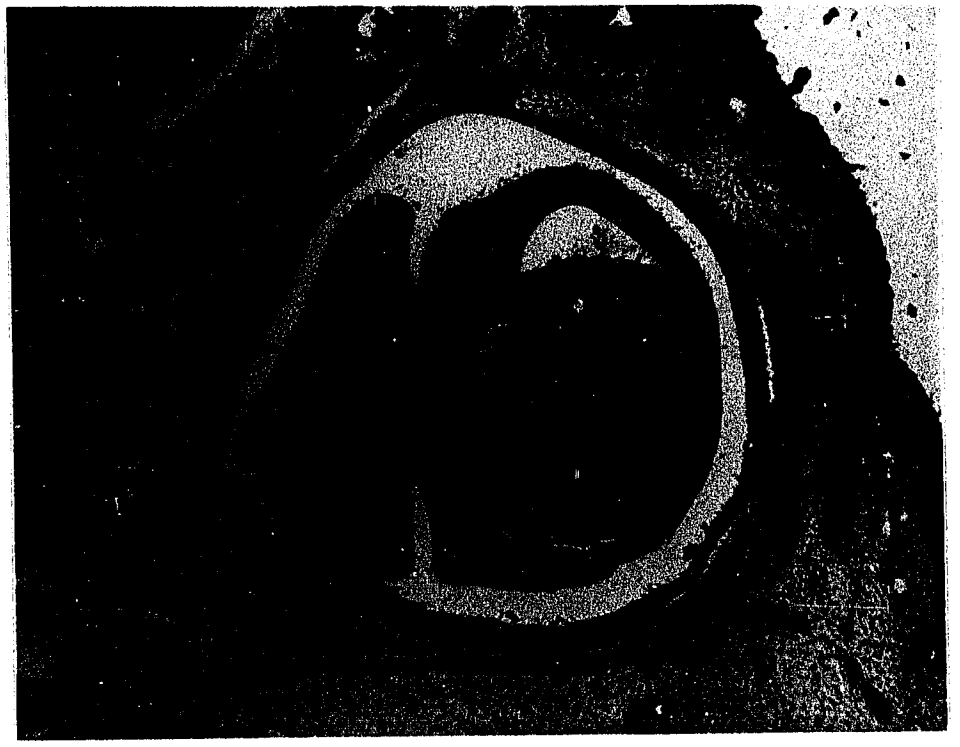
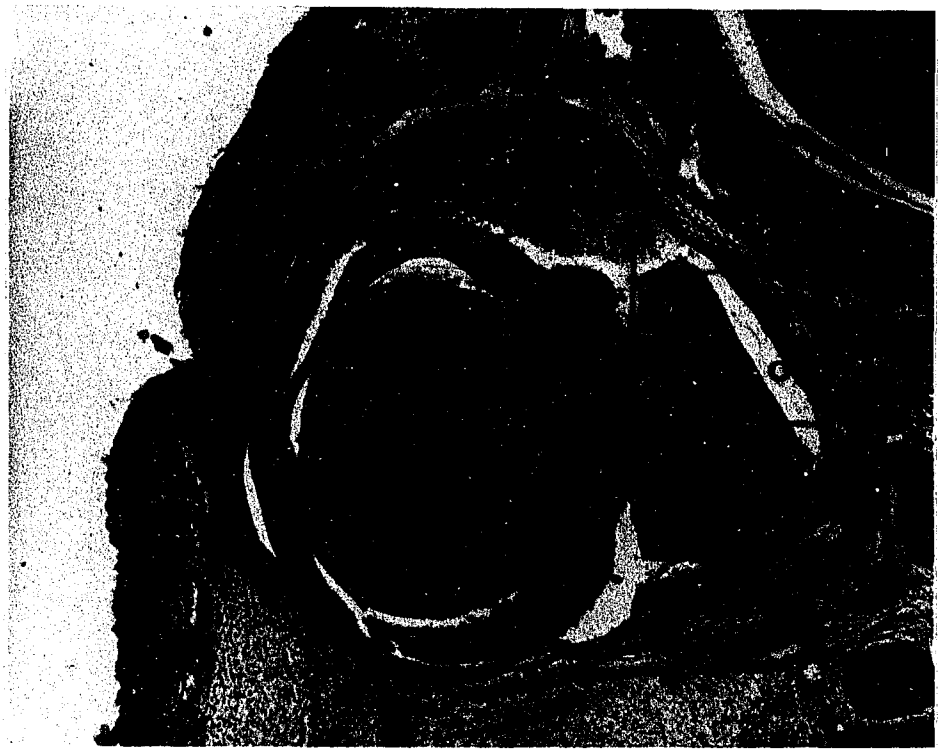


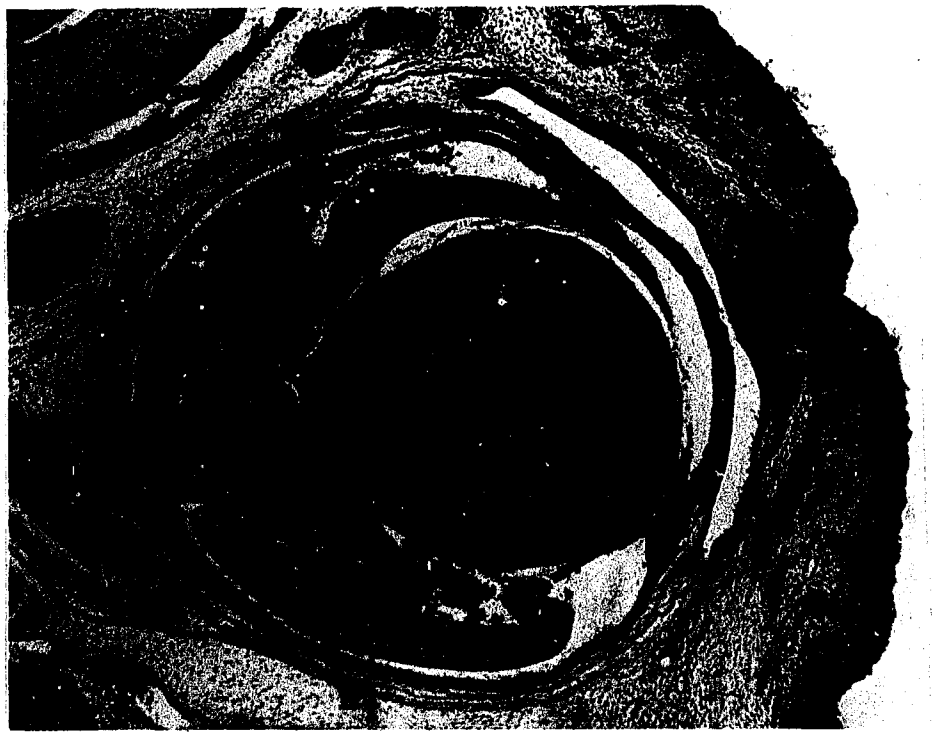
Figure 20. Section of the right eye of an abnormal day 20 rat fetus. H. and E. Approximately 45X.

Female No. 102v. Group VIII, vitamin A-free plus nitrite diet. RM indicates fibrous retrolenticular membrane connected with extraocular mesoderm; Col, coloboma; E, eversion of retina; IR, folded internal leaf of retina; Ro, rosette; HA, hyaloid artery; ON, optic nerve fibers; $H_o$, hemorrhage in space of optic vesicle.

Figure 21. Section of the left eye of an abnormal day 20 rat fetus. H. and E. Approximately 50X.

Female No. 16v. Group VIII, vitamin A-free plus nitrite diet. RM indicates fibrous retrolenticular membrane; IR, folded internal leaf of retina; Ro, rosette; $H_o$, hemorrhage in space of optic vesicle; $H_i$, hemorrhage at internal leaf of retina.





strand of connective tissue which joins extraocular mesoderm with the retrolenticular fibrous membrane. This strand is pierced by a long branching blood vessel (hyaloid vessel). In the retinal gap nerve fibers are seen on both sides of the vessel. The changes in the optic cup such as persistent fetal fissure, eversion of the retina and penetration of the cup by mesodermal tissue are characteristic of a typical coloboma.

Where the retina is folded and distinctly everted at the retinal cleft, (Figures 19, 20) there is a duplication of the internal leaf of the retina and the "innermost" layers of the everted portion are turned outward and face the choroidea. The everted retina ends abruptly at the point of junction with the external leaf of the retina. In some abnormal eyes the posterior portions of the internal retinal leaf form many folds (Figures 19, 20, 21). Some of the folds when cut transversely appear in sections as "rosettes," in which the retinal cells may be radially arranged around a variably defined inner circle (Figure 21). The space within this circle corresponds to the cavity of the fold which is a part of the lumen of the optic vesicle. In some sections, especially where retinal folds are more evident, the lumen of the optic vesicle, which is the space between the two layers of the retina, is greatly increased (Figure 20). In the areas not folded the internal leaf of the retina is of approximate normal thickness and has differentiated into two rather ill-defined layers of cells. The histologic structure of the internal leaf of the retina

shows little resemblance to that of a normal newborn rat (Figures 18, 20). Hemorrhages are frequently seen in the abnormal eyes and can be found in the optic vesicle and in the region of the retina (Figures 20, 21).

Anomalies of the anterior portions of the eyes as a result of maternal vitamin A deficiency have been described by Warkany and Schraffenberger (1946). In their work these developmental defects were almost as frequent and accompanied the changes described in this experiment. Their experimental rats were undoubtedly much more deficient in vitamin A than were the rats of this experiment as the reproductive efficiency was very much reduced in their rats. Only 36 of 90 females found to have sperm in the vaginal smear retained the products of conception later than the 13th day of pregnancy. Of these only seven mothers carried their young to term. The reproductive efficiency of the females reported here is described above. The purpose of their experimental procedure was to describe congenital malformations induced by maternal vitamin A deficiency, whereas the experiments reported here used induced congenital malformations as only one measure of the effect of ingested nitrite on reproductive performance of rats under various treatments.

Induction of fetal anomalies in rats as a result of maternal deficiency of vitamin A requires a stage of severe depletion of vitamin A. Jackson and Kinsey (1946) reporting on this subject stated that the ocular defects occur in the young rat

only when the maternal vitamin A deficiency is extremely severe, so advanced in fact, that fetal resorption is common and normal birth is impossible.

Most of the eyes serially sectioned proved normal on histologic examination. Table 10 summarizes the occurrence of ocular anomalies in fetuses in experiment II, and gives the number of days from the time that the female was placed on the vitamin A-free ration until day 20 of pregnancy.

It would appear that nitrite had an effect upon reducing maternal vitamin A stores to a level that induced anomalies. This effect may be more apparent than real. Most of the females that were in the vitamin A-free diet groups did not have as long a period of time between being placed on the vitamin A-free diet and day 20 as did the vitamin A-free plus nitrite females that had fetuses with malformations. Perhaps this period of time is not a factor as all rats on the vitamin A-free diet (groups V, VI, VII, and VIII) received 40 I.U. of vitamin A orally every ten days until day zero of pregnancy after which they did not receive any additional vitamin A. Moore (1957) reported that 4 I.U. of vitamin A are sufficient to result in optimal growth and normal blood levels of vitamin A, but storage in the liver does not occur. This should imply that as long as rats receive an average of 4 I.U. of vitamin A daily they would not need to use any stored vitamin A for normal growth. If such would be the case, then the liver vitamin A level would not decrease with increasing time on a

Table 10. Females with anomalous litters

| Litter from group | Days on vit. A-free diet | No. alive in litter | No. of resorptions | Adrenal[a] | Thyroid[a] | Pituitary[a] | Liver[b] |
|---|---|---|---|---|---|---|---|
| VIII | 226 | 4 | 1 late | ---- | --- | --- | 3.17 |
| VIII | 261 | 7 | 1 late | ---- | --- | --- | 3.66 |
| VII | 343 | 8 | 1 late | 12.0 | 8.5 | 2.5 | 2.42 |
| VII | 229 | 3 | 0 | 20.9 | 7.3 | 3.4 | 3.20 |
| VI | 266 | 2 | 5 early; 1 late | 21.5 | 8.6 | --- | 2.80 |
| VIII | 331 | 1 | 1 in process; 1 late | 10.4 | 6.7 | 1.8 | 2.24 |
| VIII | 228 | 1 | 4 late | 16.6 | 8.1 | 2.5 | 2.30 |
| VIII | 213 | 0 | 7 in process | 28.6 | 5.3 | 2.9 | 2.10 |
| Mean | | 3.25 | 2.6 | 18.3 | 7.4 | 2.6 | 2.75 |

[a]Mg./100 gm. body weight.

[b]Gm./100 gm. body weight.

vitamin A deficient diet supplemented with the necessary amount of vitamin A to maintain optimal growth. If this would be valid, then all females would have entered pregnancy with the level of liver vitamin A that was present at the time that they were placed on the vitamin A-free diet. Thus nitrite in the water would seem to have reduced the level of vitamin A to a point that anomalies were induced in some fetuses. However, if that were the case there should have been malformations induced in other females that were in the same treatment groups but became pregnant with less time elapsing between being placed on the vitamin A-free diet and day 20. This was not the case.

Most of the malformed fetuses were from litters in which the male was a nitrite male. However, it does not seem reasonable that the male was a factor in inducing these anomalies.

The average number of live fetuses in the litters with anomalies was reduced considerably from the mean number of fetuses for group VIII, which had the smallest mean number of fetuses of any treatment group (3.25 vs. 6.95, Tables 10, 19). The average number of resorptions (fetuses and sites) was greater than for any treatment group. The sum of the average numbers of fetuses and resorptions is less than that of any treatment group and would indicate impairment of reproductive efficiency.

The average weights of the adrenal, thyroid and pituitary glands of the females producing anomalous litters are greater

than the mean weights of any treatment group. The average liver weight is less than that of any treatment group. These findings would be indicative of a stress reaction in those females.

Although anomalies were induced in this experiment and were mainly induced in fetuses of females on the vitamin A-free plus nitrite ration, one could not conclusively state that nitrite invariably reduces maternal vitamin A stores so that anomalies characteristic of vitamin A deficiency result. To state this, anomalies should have been evident in other and more litters on the same regimen. When nitrite is given to female rats receiving ample dietary vitamin A, it can be concluded that congenital anomalies characteristic of maternal vitamin A deficiency will not be produced.

## Males

Males used in experiment II were euthanatized with ether at the completion of the experiment. Table 11 summarizes the mean values obtained for the variables that were observed. At the time of completion of the experiment the males were mature and approximately 1 year of age.

Methemoglobin values were zero for both groups. The rats receiving nitrite had been on treatment for nearly one year and evidently had adapted to its presence regarding reduction of methemoglobin, or it may be concluded that there may have been a faulty methemoglobin procedure. The average

PCV and hemoglobin values reflect an erythropoietic stimulation compared with the control. This would not necessarily be compatible with zero methemoglobin values. The thyroid, pituitary and adrenal gland weights are given as total weights of these glands. There was no apparent effect of nitrite on weights of these glands.

Tables of mean values for experiment II with standard errors of the means can be found in the appendix.

Table 11. Mean values obtained from male rats in experiment II

| | Diet | |
|---|---|---|
| | Control | $NO_2$ |
| Methemoglobin (gm./100 ml.) | 0 | 0 |
| Hemoglobin (gm./100 ml.) | 12.5 | 13.6 |
| Packed cell volume (%) | 41.4 | 47.0 |
| Thyroid weight (gm.) | 30.6 | 27.8 |
| Pituitary weight (gm.) | 12.4 | 12.2 |
| Adrenal weight (gm.) | 60.7 | 53.7 |

## SUMMARY AND CONCLUSIONS OF EXPERIMENTS I AND II

The effects of nitrite were evaluated in timed pregnancy rats as to its influence on hematologic, physiologic, and reproductive responses under various regimens regarding dietary vitamin A. Potassium nitrite in experiment I was given daily in the feed throughout pregnancy to females either on a commercial laboratory ration or on a vitamin A-free ration. It was given daily in the drinking water in experiment II either for at least 60 days before breeding to males and females, or given only throughout pregnancy to females on a vitamin A-free ration.

Initial and final body weight analyses were conducted. Packed cell volumes, hemoglobin and methemoglobin values were determined at various times on whole blood. The weights of maternal adrenal, thyroid and pituitary glands were determined. Liver weights and liver vitamin A analyses on pooled samples were determined. Reproductive performance regarding pregnancy rate, number and average weight of fetuses and number, position and duration of resorptions was determined. One fetus from each litter was serially sectioned through the eye region for histologic examination for the presence of congenital malformations.

Potassium nitrite (0.75 per cent) in the feed reduced feed intake and decreased final body weight, whereas, potassium nitrite in water (0.25 per cent) did not produce

significant differences in mean final body weights.

Nitrite in feed and water produced small but statistically significant increases in methemoglobin values at all sampling periods with the exception of day 20 of pregnancy in the experiment utilizing nitrite in the water when methemoglobin values were increased but not to significant levels. Hemoglobin and PCV values reflected the presence of methemoglobin at most samplings. Mean hemoglobin and PCV values decreased in most treatment groups at day 20 of pregnancy, the hemodilution being attributed to effects of advanced pregnancy. Hemodilution was more pronounced in some nitrite treated groups than in controls and was attributed to an adrenal stress response in the nitrite groups. Some groups on the vitamin A-free diet plus nitrite did not have the hemodilution effect of pregnancy on day 20. This was attributed to the hemoconcentration effect of vitamin A deficiency that was sufficient to nullify the hemodilution effects of advanced pregnancy and adrenal stress.

Nitrite caused significant increases in maternal adrenal gland mean weights in all treatment groups. Effects on thyroid and pituitary mean weights were variable. Nitrite plus vitamin A-free diet increased thyroid mean weights.

Potassium nitrite had no significant effect on mean liver weights. Liver vitamin A analyses on pooled liver samples indicated that nitrite reduced liver vitamin A a small amount in groups on commercial laboratory ration. Nitrite did not

have the same effect on pooled samples from rats on the vitamin A-free diet even when pooled samples were composed of rats that were paired as to days on the vitamin A-free diet. All liver vitamin A values were low for the vitamin A-free diet groups when compared with control ration groups.

Treatments (vitamin A-free diet and nitrite) significantly reduced pregnancy rate compared with controls in the feeding experiment. Complete resorption of litters was found only in vitamin A-free plus nitrite groups in both experiments. Usually the vitamin A-free groups had the smallest litters, and nitrite demonstrated a further reduction in litter size and average weight of the fetuses. Treatments had no significant effects on distribution of the position or duration of resorptions. Nitrite-treated males had an apparent effect on reducing litter size in all treatment groups, but the effect was not statistically significant.

Congenital ocular malformations were found mainly in vitamin A-free plus nitrite groups although they were also present in vitamin A-free diet groups. The anomalies were characteristic of vitamin A deficiency-induced malformations. The malformations were characterized by retrolenticular fibroplasia in place of the vitreous, coloboma and abnormal structure and eversion of the retina.

# PART II. TERATOGENIC EFFECTS OF EXCESS VITAMIN A IN THE DOG

# REVIEW OF LITERATURE

## Excess Vitamin A and Teratogenesis

Cohlan (1953, 1954) was the first to show that excessive intake of vitamin A during pregnancy could result in congenital anomalies in laboratory animals. Moore and Wang (1945) had reported that hypervitaminosis A produced uterine hemorrhage and reduced litter size and number in pregnant rats but did not report congenital malformations. Cohlan used Wistar female rats fed a stock ration, mated (day sperm was found = day one), and given 15,000 to 75,000 I.U. of vitamin A orally as an aqueous preparation on varying periods in gestation ranging from a single dose on one day to daily doses from the 2nd, 3rd, or 4th to the 16th day of gestation. The females were usually killed on the 20th or 21st day of gestation. The excess vitamin A during pregnancy resulted in a marked litter failure. There was a 12 per cent litter rate in the treated females and an 84 per cent litter rate in the controls. When the litters were carried to term, a variety of gross congenital malformations were produced which included exencephaly, eye malformations (including "open eye"), cleft palate, shortening of the mandible and maxilla and spina bifida with meningocele and hydrocephalus. Abnormal embryos, especially those with exencephaly, could be recognized <u>in situ</u> as their amniotic sacs were swollen with excess fluid, which was often blood-stained. The offspring of the vitamin A-treated maternal rats

exhibited gross malformations at an anomaly rate of 52 per cent. Cohlan (1954) reported that the 7th to 10th day of gestation in the rat was found to be the period critically susceptible to the teratogenic action of excess vitamin A.

These results were confirmed in rats by Giroud and Martinet (1954) who gave 20,000-35,000 I.U. of vitamin A per day orally from the 4th to the 16th days of pregnancy. Vitamin A acetate and palmitate gave similar results.

Giroud and Martinet (1956) demonstrated a differential effect on fetal development in rats by giving 60,000 I.U. vitamin A per day orally for three successive days starting treatment at several different periods of gestation. A peak in incidence of cleft palate (92 per cent) with various limb defects occurred when treatment was started on day 11 and only cleft palate (49 per cent) and cataract when started on day 14. In addition to the common malformations, treatment for three days starting on day ten also produced various abnormalities of the internal and external ear. The auricules of the external ear were like cartilaginous skeletons in the regions of the cheeks. The middle ear showed little or no development, and the ossicles showed defects.

Giroud and Martinet (1957) used hypervitaminosis A to induce exencephaly in rats in order to investigate the morphogenesis of anencephaly, which they described in detail and attributed to attrition and erosion of the exposed brain *in utero*. Deuschle *et al.* (1959) studied hypervitaminosis A-

induced skeletal anomalies of the face of rats which produce protrusion of the eyes or "open eye". The exophthalmic eyes were essentially normal in structure but protruded as a result of congenital malformation of the orbital floor.

Excess vitamin A has been shown to produce malformations in species other than the rat. Kalter and Warkany (1961) used strains of inbred mice to produce various congenital malformations. They used single oral doses of 10,000 I.U. of vitamin A. Cleft palate was the most frequent defect. Abnormalities of the pinna occurred quite often and included external ears that were absent or small, defective and placed in a low position. Visceral and skeletal anomalies were also described.

Giroud and Martinet (1959) reported that excess vitamin A given orally produced various congenital malformations in the rabbit and guinea pig. Exencephaly was produced in the offspring of female guinea pigs that had received 50,000 I.U. of vitamin A from day 10 to day 13 of gestation. Anencephaly, anophthalmia, microphthalmia, facial dysgenesis and syndactyly were congenital malformations that were produced in rabbits with doses of 125,000 to 150,000 I.U. of vitamin A from the 5th to the 10th days or the 10th to the 13th days of gestation. Higher doses resulted in abortions of the whole litter. At all levels the predominant effect was embryonic death, and malformations were relatively infrequent.

Millen and Woollam (1960) reported that an aqueous solution of vitamin A in Tween 80 given subcutaneously in adequate doses to a pregnant rat or mouse was just as effective as oral administration in producing congenital abnormalities. This method may have advantages over oral administration regarding convenience, more precise dosage and circumventing the vagaries of intestinal absorption.

Murakami and Kameyama (1965) also reported on malformations of the mouse fetus produced by hypervitaminosis A. They used a single intraperitoneal injection of 15,000 I.U. of vitamin A. In addition to numerous other malformations, they reported microtia, anotia and abnormalities of the auricle such as absence, lobulations or dislocation. The highest incidence of auricular malformations did not coincide with the highest incidence of cleft palate as to the day of treatment. Abnormalities of the tail such as short tail and curvature of the tail were observed. Treatment on day eight of pregnancy produced the highest incidence of both tail and auricular malformations. Their comment concerning the action of hypervitaminosis A was that the hypervitaminosis A exerts either a suppressive effect chiefly upon mesenchymal tissue just before its differentiation into the primordium or upon mesenchymal tissue during differentiation.

Marin-Padilla and Ferm (1965) using the golden hamster produced a variety of developmental malformations in the offspring of mothers treated on the eighth of gestation. They

removed embryos after treatment and found somite necrosis within 12 hours of the administration of vitamin A. The somite necrosis reached a maximum at 24 hours. Somite necrosis was considered to be the basic teratogenic action of vitamin A leading to such malformations as cranioschisis and sacral rachischisis which were considered to result from an abnormal axial mesoderm which caused malformations of the axial skeleton.

Giroud et al. (1956) reported that the concentration of vitamin A in fetal tissues was increased after treating female rats with large doses of vitamin A at a teratogenic stage in embryogenesis. A relatively small increase in vitamin A concentration was found, but the authors believed that vitamin A exerted a direct teratogenic effect on the embryo. Although Woollam and Millen (1963) have found increased vitamin A concentrations in offspring of vitamin A treated dams, the concentration of vitamin A is no greater in the affected embryos than in their normal siblings. They did not feel that there was sufficient indication to state that the identification of a teratogenic agent in the fetus established its effect on the fetus as direct action.

The incidence of congenital malformations from excess vitamin A can be altered by the concomitant use of other agents. Woollam and Millen (1957) and Millen and Woollam (1957) using 20 mg. of cortisone acetate increased the frequency of cleft palate and exencephaly in Wistar rats given

60,000 I.U. per day of vitamin A orally from the 8th to the 13th day of pregnancy. On the other hand, subcutaneous administration of 1.5 units per day of protamine zinc insulin from the 9th to the 12th day of gestation to females also receiving either large doses of vitamin A alone or vitamin A and cortisone virtually eliminated the occurrence of young with brain deformities (Millen and Woollam, 1958).

Cohlan and Stone (1961) reported that cortisone did not potentiate the brain malformation rate induced by hypervitaminosis A in the rat fetus. Insulin did not prevent the brain malformations. Parathyroidectomy, thymectomy or bilateral adrenalectomy on the third day of gestation did not appear to alter gross normal development or influence the incidence of hypervitaminosis A brain malformations. Kochhar (1965) also reported that cortisone did not modify the incidence of cleft palate induced by hypervitaminosis A.

Woollam and Millen (1958) also reported that the administration of 4-methyl-2-thiouracil as a 0.1 per cent solution in lieu of drinking water from the 1st through the 10th day of pregnancy in rats also receiving large doses of vitamin A greatly increased the incidence of deformities of the skull and brain. These same workers reported that the subcutaneous administration of 0.6 mg. thyroxine daily from the 8th to the 13th day of pregnancy prevented the occurrence of any congenital malformations of the head and palate in rats that received large doses of vitamin A orally on those same days (Millen

and Woollam, 1959). A mixture of B-vitamins injected subcutaneously on days 8 to 13 of pregnancy, which was the same period that large doses of vitamin A was given, greatly reduced the incidence of cleft palate and prevented any brain malformations (Millen and Woollam, 1958). The series of findings of Millen and Woollam concerning modification of teratogenic activity lead them to think that hypervitaminosis A produces its effects by interference with the carbohydrate metabolism of the developing embryo. This is not completely tenable as the agents used as modifiers directly or indirectly affect protein metabolism as well (Guyton, 1961).

Wilson (1964) investigated the interaction of teratogenic agents in Wistar-descendant rats. One of the interactions that he tested was that of hypervitaminosis A and trypan blue. Both are known teratogens. The dosage of each was that which had been determined to be a low but suprathreshold dose. The vitamin A dosage was very high, 300,000-450,000 I.U. and was given orally in cottonseed oil on the eighth day. A potentiation of effect between these two agents was demonstrated when low but suprathreshold doses of each were used. Potentiation also occurred when a low but suprathreshold dose of either agent was used in conjunction with a subthreshold dose of the other.

Härtel and Härtel (1960) reported on the teratogenic effect of audio-visual and immobilization stress in rats. The pregnant females were subjected from the 9th to the 12th day

of pregnancy to one or the other of these types of stresses or to stress combined with oral administration of 15,000 I.U. of vitamin A in oily suspension daily from the 8th to the 12th day of pregnancy. The stresses alone had no effect on congenital malformations. However, immobilization stresses potentiated the teratogenic effect of vitamin A.

The administration of a teratogenic agent to a number of pregnant mammals of a single species under identical conditions of timing and dosage does not produce a uniform effect on the development of young. In any litter the response may not be uniform. Woollam and Millen (1961) determined the influence of a single factor such as the site of implantation on the susceptibility of the mouse embryo to the teratogenic effects of hypervitaminosis A. Their findings revealed (1) that fetuses situated at the ovarian end of the uterine horn were less subject to cleft palate than those at the cervical end, and (2) that the probability of cleft palate increased as the number of young in the uterine horn increased. These findings were obtained as a result of a single subcutaneous injection of vitamin A in Tween 80 on day 11 of pregnancy.

Most of the work concerning vitamin A as a teratogen has been based on the use of large doses of vitamin A. Cohlan (1954) commonly used 35,000 I.U. in rats and Giroud and Martinet (1956) have used 60,000 I.U. of vitamin A in rats on consecutive days at different stages of pregnancy. Kalter and Warkany (1961) used single doses of 10,000 I.U. of vitamin

A in mice. Giroud and Martinet (1962) investigated the teratogenic effect of much smaller doses of vitamin A in mice. They tested doses of 500, 250, and 100 I.U. of vitamin A orally on the 8th to the 10th day of pregnancy. Only the 100 I.U. dose did not produce malformations although it provoked abortions. When these dosages were placed on an I.U./kg. basis, they were lower than dosages that have been used in human therapeutics.

Takekoshi (1964) investigated thyroid function and vitamin A teratogenesis. He reported that methylthiouracil potentiated the teratogenic effect of excess vitamin A in mice when both were administered from the 10th to the 13th day of pregnancy. Cleft palate and digital malformations were observed in the fetuses from this group. Methylthiouracil did not produce any malformations when used alone. Male rats were used to investigate a relationship between blood concentration of thyroid hormone and action of vitamin A. An intramuscular injection of 50,000 I.U. of vitamin A in Tween 80 was used for four days. It was found that vitamin A increased thyroid weight and at the same time thyroidal $I^{131}$ uptake was elevated. Serum protein bound iodine was decreased. Vitamin A plus methylthiouracil resulted in a greater increase in thyroid weight, a much reduced thyroidal $I^{131}$ uptake and a decreased serum protein bound iodine than in the rats receiving vitamin A alone. Adrenal weight was greatly increased in the rats receiving vitamin A plus methylthiouracil than in those admin-

istered vitamin A alone. Shichyo and Shimoda (1962) reported that vitamin A accelerates de-iodination of thyroxine and that the concentration of circulating thyroid hormone may be reduced as a result.

Poswillo and Roy (1965) studied the pathogenesis of cleft palate in the rat. They employed three methods for production of cleft palate. These methods were x-radiation on day 13-1/2, 50,000 I.U. of water-soluble vitamin A from the 8th to the 13th day of pregnancy plus 25 mg. cortisone acetate from the 9th to 12th day and puncture of the amniotic sac on day 15-1/2 of pregnancy. The causal mechanism involved in the vitamin A-cortisone technique was a combination of delayed palatine shelf movement plus tongue obstruction, further hampered by excessively wide palatine shelves. The palatine shelves did not exhibit fibrillogenesis until much later than normal. There was a delay in descent of the tongue and this accounted for increased tongue resistance at a time when tongue obstruction is normally absent. Normal maxillary growth throughout the period of delayed shelf movement allowed a normal increase in head width. This expansion of maxilla prevented the late-turning shelves from meeting and fusing. Shelf movement in these embryos was a simple hinge rotation without the transposition of tissue toward the midline that characterized normal plate closure.

Walker and Crain (1960) used hypervitaminosis A in mice to study the formation of cleft palate. They used 10,000 I.U.

of vitamin A in oil orally on days 11 and 12 of gestation. Strain differences in incidence of cleft palate were noted. Subdivisions of cleft palate were made on the basis of whether one or both palatine shelves were horizontal or vertical. The widest cleft was formed when the medial borders of the shelves were in a vertical plane; a cleft of intermediate width was present when one shelf was horizontal and the other vertical; the narrowest cleft was produced when both shelves were in a horizontal position. In general the position of the tongue relative to the palatine shelves varied with the location of the palatine shelves. If the shelves were vertical, the tongue was wedged between the shelves. When one shelf was vertical and the other horizontal, the tongue was over the horizontal shelf and medial to the vertical shelf. When both shelves were horizontal, the tongue would overlap the ventral surface of both shelves. This procedure of analyzing cleft palates by palatal stage was applicable only to cleft palates caused by retardation of palatine shelf movement. The condition of the palatine shelves at birth could serve as a clue to the embryological mechanism of cleft palate formation. Hypervitaminosis A produced a retardation of palatine shelf movement in the strains of mice used.

Walker (1961), using various histochemical techniques and radioautography after administration of $Na_2S^{35}O_4$, studied the distribution of mucopolysaccharides in heads of mouse embryos at the stage of palate closure. Distribution of

aldehyde fuchsin-positive material corresponded to sites of $S^{35}$ incorporation. He concluded that the ground substance of the palatine shelves contains a considerable amount of sulfated acid mucopolysaccharide. Elastic fibers were not found in the heads of 15 day embryos. Walker hypothesized a relationship between the sulfated mucopolysaccharides and various embryological events. This was based on the presence and time of appearance of sulfated mucopolysaccharides in locations where events were more easily explained by assuming involvement of a firm, elastic gel, such as gels in which sulfated mucopolysaccharides can be found. Walker and Crain (1961) postulated two causes for retardation of palatine shelf movement. One was that hypervitaminosis A interfered with biochemical processes within the palatine shelves, especially those related to sulfated acid mucopolysaccharides. The other postulated cause was increased resistance of the tongue to being displaced by the palatine shelves.

Kochhar (1965) investigated the effects of hypervitaminosis A on palate closure in the rat. He reported that there was no delay in the movement of the palatine shelves from vertical to horizontal position in the vitamin A-treated embryos. Toluidine blue staining coupled with $S^{35}$ radioautography revealed that there was no reduction in the intercellular ground substance as indicated by the sulfated acid mucopolysaccharide content. Vitamin A dosage increased the incorporation of $S^{35}$ into the embryonic tissue during the

period of palatal closure. Morphological factors were described that possibly were responsible for cleft palate. These were:

(1) reduced amount of mesenchymal tissue outfolded from the maxillary processes resulting in overly narrow palatine shelves, (2) size of the maxillary bone reduced due primarily to replacement with heterotopic cartilage, (3) the two limbs of dental laminae for upper molars, which were not lodged in maxillary bone because of defects in the latter, diverged and thus separated palatal tissue from the maxilla, (4) frequently the dental laminae for the upper molars arose from oral epithelium more medially than in controls and thus limited laterally the amount of mesenchymal tissue which could participate in the formation of the secondary palate and alveolar process, and (5) abnormal infoldings of oral epithelium appeared to entrap some of the palatal mesenchymal tissue which should have been included in the formation of the secondary palate.

## Use of Domestic Animals in Teratogenic Studies

Deformities have been produced experimentally in embryos of domestic animals. Hale (1933, 1935, 1937) reported on the congenital effects of vitamin A deficiency in pigs. Goodwin and Jennings (1958) and Palludin (1961) described congenital malformations in swine as a result of experimentally

induced maternal vitamin A deficiency.

Young (1952) experimentally produced congenital malformations in pigs by the vaccination of sows 14 to 16 days after breeding with attenuated hog cholera virus. These sows had previously been vaccinated with Boynton's tissue vaccine (B.T.V.). The most obvious malformation was ascites and edema of the body and legs. Kitchell et al. (1953) reported further on this problem. These investigators noted production of ascites, somatic edema, cephalic asymmetry, microagnathia, malformations of limbs and one anomalous right subclavian artery in porcine fetuses. The dams producing the abnormal fetuses were either non-immunized or B.T.V. immunized early in life and had received one injection of attenuated hog cholera virus when 14 to 16 days pregnant. Young et al. (1955) described further experimental work concerning this problem. Abnormalities were produced in 38 per cent of the fetuses from experimentally injected dams. Hog cholera virus originating from rabbit-modified hog cholera virus of porcine origin and virulent enough to cause death in 12-day-old susceptible test pigs was isolated from the spleens of 6 of 10 fetuses from four litters. Virus was not demonstrable in the spleens of the dams.

Ross et al. (1944) and Cunha et al. (1944) reported on the occurrence of congenital malformations such as syndactylism, talipes and paralysis agitans of nutritional origin in swine. The basal ration used was deficient in a factor or factors

necessary to support normal reproduction and lactation in the sow. Good quality alfalfa meal when added to the basal ration wholly or in part corrected the deficiencies of the basal ration. Addition of soybean lecithin and pyridoxine to the basal ration resulted in normal reproduction. Addition of other single factors of the B-complex accentuated the frequency of abnormalities.

Brum et al. (1958) reported on 601 abnormalities affecting 337 cattle. By the chi-square test of independence it was shown that general environment, as measured by gross income per cow and month of conception, was associated with the frequency of the occurrence of animals that were abnormal in some way. Animals conceived during the winter months were abnormal in greater frequencies than expected.

Low manganese intake by gestating cows has been associated with the experimental production of calves exhibiting deformities (Dyer et al., 1964; Rojas et al., 1965). The deformed calves exhibited enlarged joints, stiffness, twisted legs, shortened humeri and a general physical weakness. The authors suggested a dietary manganese requirement for the cow of 20 p.p.m.

Binns et al. (1961) studied the etiology of a congenital deformity in calves, commonly called "crooked calves", in a feeding trial employing heifers. Two animals fed lupine and lead gave birth to full-term crooked calves while three heifers fed lupine alone gave birth to full-term normal calves.

Animals fed lead only produced normally developed calves.

Binns et al. (1963) reproduced a congenital cyclopian-type malformation in lambs by feeding under controlled conditions Veratrum californicum in both fresh and green-dried forms. This condition had occurred in epidemic proportions in certain bands of range sheep. When the ewes ingested the plant from the 1st to the 10th day of their gestation period, the fetus was not affected. Ingestion of the plant from the 1st to the 15th day of gestation caused cyclopian-type deformities to occur in lambs. When ingestion was continued after the 15th day, an abnormally high number of ewes tended to recycle. All fetuses obtained from these ewes prior to their recycling had undergone fetal death and were severely deformed.

Binns et al. (1963) determined that the 14th day of gestation of ewes was the critical time for development of congenital malformations. Ewes fed the plants on other days had normally developed fetuses or normally developed embryos which died later. The dosage of Veratrum californicum that had a teratogenic effect was less than that necessary to cause clinical signs of poisoning in ewes. Keeler and Binns (1964) reported on the extraction of chemical compounds from Veratrum californicum which had teratogenic activity.

Younger (1965) reported the delivery of a lamb with congenital malformations from a ewe that was on a chronic toxicity experiment utilizing apholate. Apholate is a polyfunctional

alkylating agent that had produced skeletal defects when injected into the yolk sac of embryonating chicken eggs. The ewe had received 195 daily doses of apholate before conceiving and continued to receive it through gestation. Lambs born to three other ewes on the study were normal. The malformed lamb showed a small deformed cranium, anophthalmia, absence of orbital cavities, shortened upper jaw, absence of patent external nares, protruding tongue, oral epithelium covering the lower incisor teeth, abdominal herniation, shortened tail and generalized edema.

James et al. (1966) investigated the effects of sublethal doses of certain minerals on pregnant ewes and fetal development. One of two ewes given bismuth subtartrate for the first 45 days of gestation aborted; whereas, the other gave birth to a deformed lamb. However, the same dosage level of bismuth subtartrate given to two other ewes throughout gestation was without effect on the lambs.

There are few reports concerning the use of the canine in the experimental production of congenital malformations. Cahan (1964) comments that because of placental similarities with man, experiments should be conducted on dogs, which, however, are rarely used.

Knouff et al. (1936) used pregnant bitches to investigate the etiology of mottled enamel of deciduous teeth. They demonstrated that in the bitch the placenta is permeable to the fluorine ion. Sections through the molar teeth of a fetus

aborted from toxic sodium fluoride levels showed marked interference in enamel formation.

Friedman (1957) reported on the experimental effect of O-diazoacetyl-L-serine (azaserine) on pregnancy of the bitch. Azaserine inhibits purine synthesis which results in inhibition of nucleic acid synthesis. Bitches were bred on two consecutive days during the 11th to 17th day from the start of proestrus and were recorded as zero days pregnant from the time of the first mating. Azaserine at 5 mg./kg. of body weight was given intramuscularly daily for three days. Fetal development ceased in 13 of 14 pregnant bitches treated from the 19th to the 23rd day of pregnancy. One pup survived of six allowed to remain after a partial hysterectomy on the 27th day of gestation.

Friedman as cited in Murphy (1960, p. 110) treated bitches with 6-diazo-5-oxo-L-norleucine (DON) with the result that resorptions, cleft palate, umbilical hernia, abnormal caudal vertebrae and abnormal maxillary, mandibular and digital bones were produced. These effects were produced when the pregnant bitch was treated on days 20, 21 and 22 of gestation. DON is a structural analogue of glutamine. It antagonizes glutamine in some systems with a resultant inhibition of purine synthesis. The toxic effect on the rat embryo is partially prevented by adenine and guanine (Murphy, 1960).

Thalidomide has been used experimentally in pregnant bitches. (Weidman et al., 1963). Bitches were mated on two

successive days. Thalidomide was given from day 1 or 5 through day 21 or 25 of gestation. Uncompleted pregnancy and neonatal mortality were more frequent following thalidomide administration than had been observed in the same bitches prior to the study. The most common abnormality was malformation of the tail vertebrae. A still-born pup had cleft palate. One pup had a supernumerary rib, and another pup had abnormal sternebrae.

# MATERIALS AND METHODS

## Animals

Healthy Beagle bitches that had been previously used for establishing a disease-free dog colony were housed in departmental quarters. The animals were housed in inside pens and fed a commercial dry dog food[1] and water ad libitum.

## Method of Handling

All bitches had produced normal litters previously. Twelve females were mated two times at early to mid-estrus with one day between matings. They were recorded as zero days pregnant from the day between the two matings. Evans and Cole (1931) found that ovulation occurred within 24 hours of the first acceptance. Gier (1950) reported that ovulation occurred at approximately the middle of the estrus period and fertilization was delayed several days pending completion of maturation. Griffiths and Amoroso (1939) reported that ovulation takes place 1 to 3 days after first acceptance. Friedman (1957) mated bitches on two consecutive days from the 11th to 17th day from the start of proestrum.

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[1]Wayne Dog Food, Krummettes, Allied Mills, Inc., Chicago, Illinois.

Vitamin A palmitate[1] at 250,000 I.U./gm. stabilized with gelatin and sugar was given orally in No. 12 gelatin capsules in doses from 50,000 to 125,000 I.U. of vitamin A/kg. of body weight daily at varying periods of gestation from day 9 through day 31. Whelping was allowed to occur normally in most instances. Cesarean section was performed on four bitches when indications of whelping were evident. Observations were made on the state of the uterine contents at that time.

## Examination of the Pups

The pups were examined grossly for evidence of congenital malformations. Some of the pups were euthanatized with ether and examined for internal gross malformations.

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[1]Palmilets, supplied by Chas. Pfizer and Co., Inc., New York, New York.

## RESULTS AND DISCUSSION

Daily doses of vitamin A below 125,000 I.U./kg. were non-teratogenic with one exception (Table 12). The exception was a dosage schedule of 50,000 I.U. of vitamin A/kg. of body weight given daily from day 17 through day 27 of gestation. A litter of five pups was whelped on the 60th day of gestation; of these two were normal and three had minor deformities. One pup had accessory auricular structures and two had unilateral posterior extension of the commissure of the lips which involved the right side in both. Other gross abnormalities were not evident. This same bitch was treated during a subsequent pregnancy with 112,500 I.U. of vitamin A/kg. of body weight from day 17 through 22 of pregnancy without resulting in any malformations of the offspring. The gestation length of the pregnancy resulting from this breeding was 64 days, whereas the average gestation length in this group of bitches was 60.6 days. Eckstein and Zuckerman (1956) indicated that the period of gestation in dogs generally ranges from 58 to 63 days but owing to the uncertainty in the timing of ovulation its length may be difficult to calculate accurately. If this bitch had been bred too early in estrus, the actual gestation period could have been less than that recorded. If this occurred, treatment with vitamin A would have occurred prior to a susceptible stage of organogenesis. Implantation of the blastocyst in the bitch is said to occur on

Table 12. Results of administration of excess vitamin A to pregnant bitches

| I.U. vit. A/kg. (days, incl.) | Female No. | Gestation length in days | Litter size | Abnormalities |
|---|---|---|---|---|
| 50,000; day 17-27 | 3 | 60 | 5 (2 normal) | 1 with accessory auricular structures; 2 with unilateral posterior extension of the commissure of the lips |
| 75,000; day 17-28 (missed day 21) | 9 | 59 | 7 (all normal) | ---------- |
| 100,000; day 17-29 (missed day 20) | 11 | 57 | 6 (all normal) | ---------- |
| 100,000; day 17-29 | 6 | 59 | 2 (all normal) | ---------- |
| 112,500; day 17-22 | 3 | 64 | 4 (all normal) | ---------- |
| 125,000; day 9-14 | 1 | 61 | 4 (all normal) | all in one horn of uterus |
| 125,000; day 12-22 | 6 | 58 | 5 (2 normal) | 2 with cleft palate, accessory auricular structures, kinked tail; 1 with accessory auricular structures |

Table 12. (Continued)

| I.U. vit. A/kg. (days, incl.) | Female No. | Gestation length in days | Litter size | Abnormalities |
|---|---|---|---|---|
| 125,000; day 17-22 | 9 | 60 | 5 (4 normal) | 1 with accessory auricular structures |
| 125,000; day 17-22 | 11 | 59 | 6 (0 normal) | 1 stillborn; 3 others with cleft palate, kinked tail; 2 of which had very "clubbed" ears; 6 with accessory auricular structures; 1 with tags on face |
| 125,000; day 20-29 | 10 | 63 | 3 (alive) 2 (mummified) | 2 mummified with cleft palate; 3 alive with cleft palate, accessory auricular structures; female also with kinked tail, unilateral renal agensis with absence of that horn of the uterus<br><br>large amount of sanguinous amniotic fluid |
| 125,000; day 22-29 | 4 | 67 | 5 (3 normal) | 2 with accessory auricular structures |
| 125,000; day 24-31 | 2 | 60 | 10 (all normal) | reduced litter to 4 which subsequently died in next 72 hours; no anomalies |

the 18th day after ovulation (Gier, 1950). In the greyhound bitch the blastocysts implant 16-1/2 to 17 days after ovulation (Griffiths and Amoroso, 1939). The embryologic stage at the time that an agent acts determines which tissues are susceptible to teratogenesis and this greatly determines the type and incidence of deformity produced (Wilson, 1961). Before the implantation of the blastocyst a teratogen can kill the embryo but is powerless to deform it (Woollam, 1965). Treatment was recorded from day 17 of the pregnancy through day 22 but could have been from day 13 through day 18 as regards the blastocyst and thus could have occurred prior to a susceptible embryologic stage.

Daily treatment of bitch No. 9 with 75,000 I.U. of vitamin A/kg. of body weight on day 17 of pregnancy through day 28, with treatment on day 21 missed, resulted in a litter of seven normal pups whelped on the 59th day of gestation (Table 12). Treatment of this same bitch during a subsequent pregnancy six months later with 125,000 I.U. of vitamin A/kg. of body weight from day 17 of the pregnancy through day 22 resulted in a litter of five pups whelped on the 60th day of gestation (Table 12). Four of the pups were normal; one had accessory auricular structures. The length of gestation during these two pregnancies was practically the same (59 days and 60 days). The treatments were initiated in both cases on day 17 of pregnancy. The treatment schedule at the lower level of vitamin A extended over a longer period of gestation than did

the treatment at the higher level (through days 28 and 22, respectively). However, treatment on day 21 of the pregnancy at the lower level did not occur, whereas it did occur at the higher level. If day 21 would have been the susceptible time for inducing malformations in auricular structures, the teratogen, i.e., hypervitaminosis A, was not present at a teratogenic level at that time. It would appear that the 75,000 I.U. vitamin A level is a subthreshold teratogenic level for bitch No. 9 and her offspring. Furthermore, the treatment schedule for bitch No. 9 of 125,000 I.U. of vitamin A/kg. of body weight daily from day 17 of pregnancy through day 22 resulted in only one pup with malformations and these were only accessory auricular structures, whereas other bitches treated with that level and through those days of pregnancy produced litters with more severe types of malformations (Table 12).

It would then seem that the offspring of bitch No. 9 were of a genotype relatively free of deformities, for teratogenic agents are said to act often, if not always, in a complementary fashion with the genotype of the embryo to produce malformations (Wilson, 1961). This is most evident in strains of laboratory animals which without any treatment exhibit a low incidence of a "spontaneous" malformation and when subjected to a teratogenic agent show a marked increase in this incidence. Fraser and Fainstat (1951), Fraser et al. (1957) and Kalter (1954) observed strain differences in mice in the production of cleft palate as a result of cortisone injections.

Kalter (1954) performed an experiment using mice and demonstrated that the uterine environment as well as genotype of the embryo was important in determining cleft palate after cortisone injection. A dose of cortisone into pregnant strain A mice resulted in cleft palate in 100 per cent of the offspring while the same dose into pregnant strain C57BL produced offspring with only 19 per cent cleft palate. When the two strains were crossed so that C57BL males bred with A females, 43 per cent of the young had cleft palate. However, when the cross was reversed, i.e., a C57BL female with an A male, the incidence of cleft palate dropped to only 4 per cent.

Not only are the time at which an agent acts and the genotype of the embryo important in determining the pattern of malformation, but also, teratogenic agents often produce patterns of malformations which are characteristic of a particular agent as a consequence of interference with a particular phase of metabolism (Wilson, 1961). Certain patterns of defects produced by different agents are often strikingly similar, which would indicate relatedness in the pathways of action (Runner, 1959). Giroud and Martinet (1956) found that the teratogenic effect of hypervitaminosis A in rats depends on the days of gestation on which treatment was carried out.

Daily treatment of bitch No. 6 with 100,000 I.U. vitamin A/kg. of body weight from day 17 of pregnancy through day 29 resulted in litter of only two normal pups whelped on the 59th

day of gestation (Table 12). The bitch was not observed during parturition. This same bitch was treated during pregnancy seven months earlier with a treatment schedule of 125,000 I.U. vitamin A/kg. of body weight daily from day 12 of pregnancy through day 22 which resulted in a litter of five pups whelped on the 58th day gestation, two of which were normal (Table 12). Two severely malformed pups had cleft palate, accessory auricular structures and kinked tails that were affected near the distal end. The other affected pup had accessory auricular structures.

Two pups in a litter would indicate reduced litter size in view of the fact that the same bitch had a litter of five in an earlier pregnancy during this experiment. The litter of two was associated with a treatment schedule of 100,000 I.U. of vitamin A that extended from day 17 of pregnancy through day 29, whereas the litter of five was associated with a treatment schedule of 125,000 I.U. of vitamin A that extended from day 17 of pregnancy through day 22. The longer treatment time associated with the litter of two puppies could be a factor in reduction of the litter size, although, like malformations, mortality decreases as embryonic age at the time of teratogenic treatment increases. Cohlan (1953, 1954) found marked litter failure and reduced litter size in rats that were treated with 15,000 to 75,000 I.U. vitamin A during various days and for varying periods of gestation.

Bitch No. 6 following the 100,000 I.U. treatment schedule was not observed during whelping. Therefore, the possibility exists that there could have been mummified malformed fetuses delivered and subsequently eaten by the bitch during the natural act of removing products of parturition. Early embryonic death could also account for reduced litter size as could reduction in viable ova. Bitch No. 10, which was delivered by Cesarean section on the 63rd day of pregnancy, had two small mummified severely malformed fetuses in addition to three live severely malformed pups (Table 12). This female had been on a treatment schedule of 125,000 I.U. vitamin A/kg. of body weight daily from day 20 of pregnancy through day 29. It is probable that had this bitch been allowed to deliver naturally that the mummified fetuses would have been eaten and thus not observed and recorded as products of the pregnancy. Intra-uterine mortality tends to vary directly with the rate of malformation in a number of experiments (Giroud and Martinet, 1954; Millen and Woollam, 1957; Wilson and Barch, 1949; Wilson, 1961). This was evident in the litter from bitch No. 10 which consisted of three severely malformed pups and two severely malformed mummified fetuses, whereas, only two pups from bitch No. 6 following the 100,000 I.U. treatment schedule would indicate the probability of intra-uterine mortality. There were no malformed pups in the products of pregnancy that were available for examination after the whelping of bitch No. 6. However, Giroud and Martinet (1958) using excess

vitamin A in pregnant rabbits found that regardless of the dose the predominant effect was embryonic death or abortion. Malformations were relatively infrequent, although this is not the case with most teratogenic agents and species regarding the use of excess vitamin A.

When intra-uterine death and rate of malformations vary directly, it could indicate different manifestations of the same primary injury. When embryonic stages are examined after teratogenic treatment, many severe types of malformations are found which are rarely seen if the pregnancy is allowed to go to term, for then fetal death with subsequent resorption and/or abortion will remove from observation those fetuses that are so severely malformed that they cannot survive a full term pregnancy (Wilson and Warkany, 1949; Wilson et al., 1953). This was certainly evident in the pregnancy of bitch No. 10 delivered by Cesarean section. A teratogenic agent which may produce embryonic death may also cause malformations. Such an agent might also have the effect of causing resorption, inducing malformations incompatible with intra-uterine existence or causing abortion of an embryo which might otherwise have survived and been born as a live deformed offspring (Woollam and Millen, 1963).

Cesarean section on bitch 10 also was rewarding with regard to the observation concerning the large amount of amniotic fluid associated with the three live but severely malformed pups. Cohlan (1954) reported that abnormal rat fetuses,

especially those with exencephaly, could be recognized *in situ* as their amniotic sacs were swollen with excess fluid. Polyhydramnios has been noted to occur frequently in human pregnancies that result in malformed children and to be associated particularly with certain congenital malformations such as anencephaly and esophageal atresia (Moya *et al.*, 1960). Gulienetti *et al.* (1962) used a radioisotope dilution method to measure amniotic fluid volumes of rat and mouse fetuses that had been subjected to teratogenic techniques. In craniorachischitic rats and in mice from riboflavin deficient mothers the amniotic volume was increased relatively and absolutely as related to fetal size.

The three live pups from bitch No. 10 had cleft palate, as did the mummified fetuses, and accessory auricular structures. In addition, the tail of the female was kinked near the distal end. The female pup also had left unilateral renal agenesis with an accompanying absence of that horn of the uterus. The ureter was absent; the ovary was present (Table 12). McLarland and Deniz (1961) reported on renal agenesis in the canine and hypothesized that renal agenesis may be due to non-hereditary causes induced by several factors, such as alteration of *in utero* environment, hypoxia, avitaminosis and defective ovum. They noted that the male was affected more frequently than the female and that the left kidney was involved more than the right.

Cesarean section was also performed on bitch No. 1. This female had been treated daily with 125,000 I.U. of vitamin A/kg. of body weight from day 9 of pregnancy through day 14. Indications of whelping were imminent on the 61st day of gestation when surgery was performed. Four normal pups were delivered (Table 12). All were in one cornu of the uterus indicating the probability that there had been embryonic deaths in the other cornu. In the bitch ova enter the uterus six to eight days after ovulation (Griffiths and Amoroso, 1939). Very shortly after entering the uterus the blastocysts become spaced more or less evenly throughout its length with a marked tendency to equality of distribution of embryos between the two uterine cornua. The spacing and orientation take place prior to establishment of permanent contact with the uterine mucous membrane and by the 15th day uterine swellings are noticeable (Amoroso, 1952). Treatment of bitch No. 1 was from day 9 through day 14 and thus was prior to implantation which occurs 18 days after ovulation (Gier, 1950). The dose of vitamin A was a teratogenic dose (Table 12), but congenital malformations were not produced as a teratogenic agent administered before the embedding of the blastocyst may kill the embryo (which seemed to have occurred) but does not deform it (Woollam and Millen, 1963). Rapid processes of differentiation in the embryo begin once implantation of the blastocyst has taken place and end when differentiation of the organs has been completed, i.e., when the embryo becomes a fetus (Keberle

et al., 1965).

Bitch No. 11 produced two litters (Table 12). During the first gestation she was treated with 100,000 I.U. of vitamin A/kg. of body weight daily from day 17 of pregnancy through day 29 with the exception of day 23 when treatment was missed. A litter of 6 normal pups was whelped on the 57th day of gestation. This bitch was treated during a subsequent pregnancy one year later with 125,000 I.U. vitamin A/kg. of body weight daily from day 17 of pregnancy through day 22. A litter of six pups, none of which were normal, was whelped on the 59th day of gestation (Table 12). Treatment from day 17 through day 22 of gestation was common to both pregnancies and gestation length was very nearly the same (57 days and 59 days). However, levels of vitamin A administered were different during the two pregnancies. It would seem that the genotypes of the embryos of this bitch were such that only the higher level of vitamin A was teratogenic (Wilson, 1960).

It has been suggested by Fraser et al. (1957) that a majority of spontaneously occurring malformations is the result not of a single genetic or a single extrinsic factor, but a combination of many genetic and environmental factors. The use of multiple agents in small doses has been studied to some extent and has usually involved two or more extrinsic agents at minimal levels used simultaneously. Woollam and Millen (1957a, 1957b) reported that cortisone acetate potentiated the action of excess vitamin A in producing cleft palate and

exencephaly in rats. However, Cohlan and Stone (1961) and Kochhar (1965) reported that cortisone did not modify the incidence of cleft palate produced in rats by hypervitaminosis A.

Bitch No. 11 had been treated during the pregnancy between those reported here with 75,000 I.U. of vitamin A orally and 4.4 mg. of prednisolone I.M./kg. of body weight daily from day 17 of pregnancy through day 29 (Wiersig, 1965). These levels had been determined to be non-teratogenic when used separately. However, when used simultaneously, fetal abnormalities resulted. All of the litter of three puppies from this pregnancy had umbilical hernias. Neither cleft palate, auricular abnormalities nor kinked tails were produced as a result of the vitamin A and prednisolone treatment. However, the level of 125,000 I.U. of vitamin A/kg. of body weight produced very severe abnormalities in the pups that were of a different nature from those produced by treatment of the same bitch with vitamin A and prednisolone. Auricular abnormalities were evident in all 6 of the pups in the litter from bitch No. 11 following the 125,000 I.U. vitamin A treatment schedule (Table 12). The first puppy also was stillborn. Three pups had cleft palates (Figure 22) and kinked tails (Figure 23). Two of them also had severe auricular abnormalities (Figure 24). The cleft palates, auricular abnormalities and kinked tails were typical of those seen in other litters exhibiting these anomalies. The photographs depicting these

Figure 22. Cleft palate produced as a result of treatment of a pregnant bitch with excess vitamin A.

Three of a litter from bitch No. 11 treated with 125,000 I.U. vitamin A/kg. of body weight from day 17 through day 22. Left pup has both palatal shelves horizontal. Center pup has one palatal shelf vertical and one palatal shelf horizontal. Right pup has both palatal shelves vertical.

Figure 23. Kinked tails produced as a result of treatment of a pregnant bitch with excess vitamin A. Litter from bitch No. 11. See Figure 22.

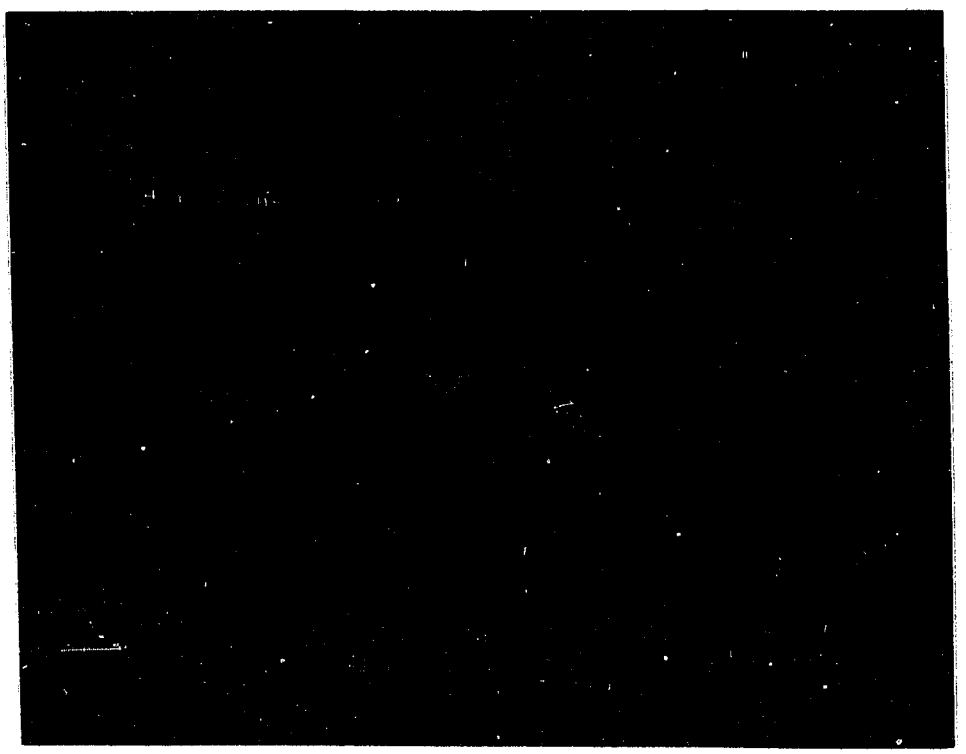
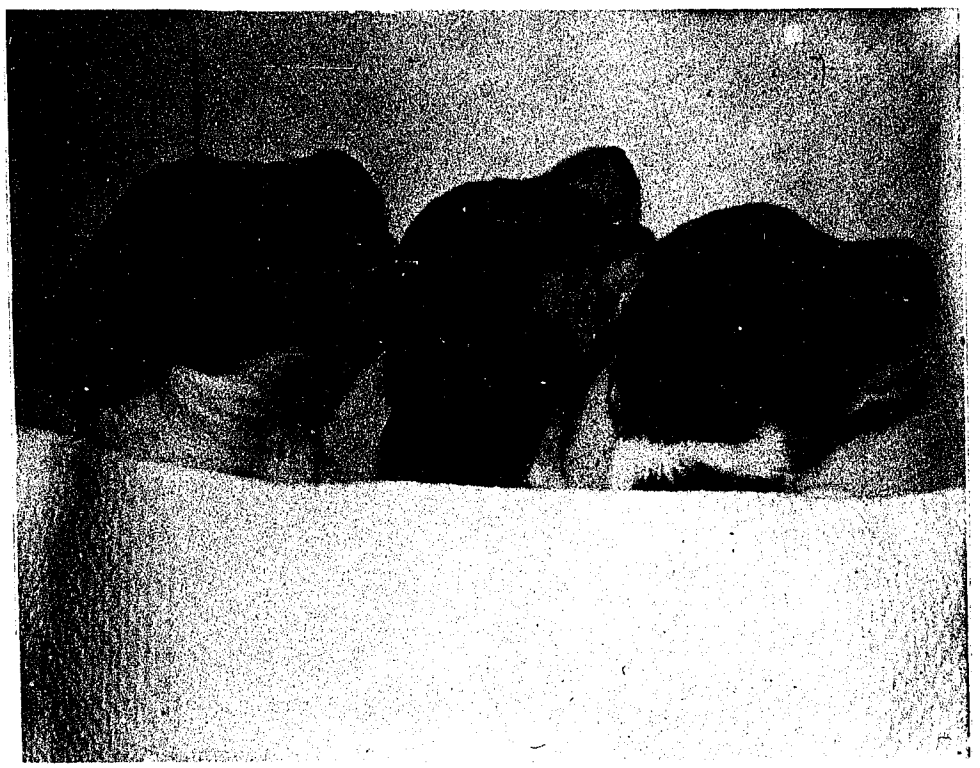


Figure 24. Auricular abnormalities produced as a result of treatment of a pregnant bitch with excess vitamin A.

Litter from bitch No. 11 treated with 125,000 I.U. vitamin A/kg. of body weight from day 17 through day 22. Pup on the left has a relatively normal ear with exception of the accessory flap as indicated. Note the thickened, bissected and accessory auricular structures on the other two pups. Skin tags are indicated on the face of the center pup.



anomalies are thus all from this litter. The pup that was most severely affected also had skin tags on the face. The severe ear abnormalities could be described as "clubbed" ears. The auricles consisted of a number of stubby flaps. One pup had a kinked tail in addition to minor auricular abnormalities. It seems in regard to this animal that the type of congenital malformation produced in the offspring may depend upon the teratogenic agents used.

Bitch No. 4 was treated with 125,000 I.U. vitamin A/kg. of body weight daily from day 22 of pregnancy through day 29. A litter of five pups was whelped on the 67th day of pregnancy (Table 12). Two of the pups had minor accessory auricular structures and the others appeared normal. Based on the length of gestation this bitch may have been mated too early in estrus, although spermatozoa cannot remain capable of fertilization for extended periods of time. However, viable spermatozoa have been recovered from the genital tracts of bitches four days after copulation (Harrop, 1960). It seems that either the progenies of this bitch are relatively resistant to teratogenic effects of excess vitamin A, or, that if the gestation period was of true length, then the treatment was administered at a time other than a susceptible period of organogenesis except that involving auricular structures.

Treatment of bitch No. 2 with 125,000 I.U. vitamin A/kg. of body weight daily from day 24 through day 31 of gestation resulted in a litter of ten pups whelped on the 60th day of

gestation (Table 12). The pups did not exhibit any congenital malformations, although four pups allowed to remain with the bitch subsequently died within 72 hours. Cause of death could not be determined. It seems that this bitch was subjected to treatments at a time when susceptibility to teratogenesis had decreased. Susceptibility to teratogenesis decreases as differentiation proceeds (Wilson, 1961; Fraser and Fainstat, 1951).

A summary of the teratogenic effects of excess vitamin A in this experiment is given in Table 13. Fifty per cent of the litters contained pups with external congenital anomalies. Sixty-two per cent of the pups in the anomalous litters displayed malformations.

It is an established fact that acquired anomalies are a function of the stage of development in which an injurious environmental agent is active. The minimal effective dose is also an important aspect of stage specificity. Embryonal tissues will exhibit sensitivity at stages adjacent to that of maximal susceptibility when exposed to large doses of a teratogenic agent, thus leading to overlapping of critical periods and confusion of anomalies produced (Cohlan, 1954). Critical stages can be most easily determined in animals in which the embryonal age is known with greater certainty than in the canine. Various times for ovulation in relation to the first acceptance of the bitch are given by different authors (Griffiths and Amoroso, 1939; Evans and Cole, 1931;

Table 13. Litter effects of excess vitamin A

| Total litters | Litters without abnormalities | Litters with abnormalities | Abnormal litters | |
|---|---|---|---|---|
| | | | Normal pups | Abnormal pups |
| 12 | 6 (33 pups) | 6 (29 pups) | 11 (38%) | 18 (62%) |

Gier, 1950). Thus difficulty would be experienced in assessing the effect of a single brief exposure of a teratogenic agent in the canine.

Results obtained from this experiment relating to period of gestation and minimal dose are given in Table 14. Daily doses of vitamin A below 125,000 I.U./kg. of body were without teratogenic effect with the exception of one anomalous litter produced as a result of 50,000 I.U./kg. of body weight from day 17 through day 27 of pregnancy. Daily doses of 125,000 I.U./kg. of body were teratogenic with the exception of one litter treated from day 9 through day 14 of pregnancy. This would have been before implantation and before the period of susceptibility to a teratogenic agent.

The distribution of abnormalities as a result of administration of excess vitamin A to the pregnant bitch is given in Table 15.

Malformations involving the auricles were produced with the highest incidence in this study. Hypervitaminosis A in

Table 14. Teratogenic effects of vitamin A in varying doses and at varying periods in gestation

| Vitamin A intake I.U./kg. | Gestation days adm. (incl.) | No. of litters | No. of pups | Abnormal pups | % Abnormal |
|---|---|---|---|---|---|
| 50,000 | 17-27 | 1 | 5 | 3 | 60 |
| 75,000 to 112,500 | 17-29 | 4 | 19 | 0 | 0 |
| 125,000 | 9-14 | 1 | 4 | 0 | 0 |
| 125,000 | 12-22 | 1 | 5 | 3 | 60 |
| 125,000 | 17-22 | 2 | 11 | 7 | 64 |
| 125,000 | 20-29 | 1 | 3 | 3 | 100 |
| 125,000 | 22-29 | 1 | 5 | 2 | 40 |
| 125,000 | 24-31 | 1 | 10 | 0 | (Did not survive) |

Table 15. Distribution of abnormalities

| Daily vitamin A intake I.U./kg. | Lips | Auricular structures | Kinked tail | Cleft palate |
|---|---|---|---|---|
| 50,000 to 112,500 | 2 | 1 | - | - |
| 125,000 | - | 3 | 2 | 2 |
| 125,000 | - | 1 | - | - |
| 125,000 | - | 6 | 3 | 3 |
| 125,000 | - | 3 | 1 | 3 |
| 125,000 | - | 2 | - | - |
| | 2 (6%) | 16 (50%) | 6 (19%) | 8 (25%) |

rodents has also resulted in congenital malformations of the external ears in addition to other anomalies (Giroud and Martinet, 1956; Kalter and Warkany, 1961; Murakami and Kameyama, 1965). The following defects of the external ears were reported: absent with placement of auricular structures in the region of the cheeks; absent or small, defective and placed in low position; microtia, anotia, lobulations and dislocations of the auricles.

Thalidomide given to pregnant dogs from day 1 of pregnancy through day 21 produced malformations of the tail vertebrae (Weidman et al., 1963). The tails were short and kinked with hairless necrotic tips that had the appearance of tissue which had been deprived of a blood supply. The tail anomaly produced by hypervitaminosis A was not of a like nature. The tails were of normal length but had a kink close to the end. Necrosis was not present (Figure 23). Abnormalities of the tail such as short tail and curvature of the tail have been reported in mice as a result of excess vitamin A (Murakami and Kameyama, 1965).

The cleft palates produced by 125,000 I.U. vitamin A dosage schedule to bitch No. 11 could be classified according to the subdivisions that Walker and Crain (1960) used in mice. Each of the three puppies with cleft palate could be placed in a different subdivision. These subdivisions were made on the basis of whether one or both palatine shelves were horizontal or vertical. In general, tongue position varied with

location of the shelves. The widest cleft was formed when the medial borders of the shelves were in a vertical plane (Figure 22, pup on right). The tongue was wedged between the shelves. A cleft of intermediate width was formed when one shelf was horizontal and the other vertical (Figure 22, pup in center). The tongue was over the horizontal shelf and medial to the vertical shelf. The narrowest cleft would be produced when both shelves were in a horizontal position (Figure 22, pup on left). The tongue overlapped the ventral surface of both shelves. These authors concluded that this procedure of analyzing cleft palates by palatal stage was only applicable to cleft palates caused by retardation of palatine shelf movement. Thus, condition of the palatine shelves at birth could serve as a clue to the mechanism of cleft palate formation. It would seem that hyper-vitaminosis A in the canine produced a retardation of palatal shelf movement as it did in the strains of mice used by Walker and Crain (1960).

The treatment regimen that produced cleft palate was 125,000 I.U. vitamin A/kg. of body weight daily from day 12 through day 22 (bitch No. 6), day 17 through day 22 (bitch No. 11) and day 20 through day 29 (bitch No. 10) as presented in Table 12. Lengths of the gestation periods were 58 days, 59 days and 63 days, respectively. Treatments with this level of vitamin A that terminated before day 17 or began after day 22 did not result in cleft palate production (Tables 14, 15). Therefore, under the conditions of this experiment the critical

period of fetal susceptibility for the production of cleft palate by excess vitamin A would be day 17 through day 22.

If ovulation could be critically timed, treatment could probably be delayed until day 18. Friedman as cited in Murphy (1960, p. 110) produced cleft palate in the pups of a bitch treated on day 20 through 22 of gestation with diazo-oxo-L-norleucine (DON). Whelping was day 60 of gestation.

Cohlan (1954) found that in 200 gram rats 25,000 I.U. of vitamin A from day 7 through day 10 were teratogenic and produced cleft palate. This would be 125,000 I.U. vitamin A/kg. This is comparable to results of Giroud and Martinet (1954). Single oral doses of 10,000 I.U. vitamin A have produced cleft palate in mice (Kalter and Warkany, 1961). This would be approximately 400,000 I.U./kg. Giroud and Martinet (1962) reported the effects of much smaller doses of vitamin A when given to mice on the 8th to the 10th day of pregnancy. Doses of 250 and 500 I.U. were teratogenic and reduced littering and 100 I.U. only induced abortions. Doses of this magnitude on an I.U./kg. basis have been exceeded in human therapeutics. The highest dosage of vitamin A used in this experiment was a minimal teratogenic dosage for inducing cleft palate formation in the canine. On an I.U./kg. of body weight basis the canine has a susceptibility to cleft palate production comparable to that of rats. Hypervitaminosis A in rats produces exencephaly that was not produced in the canine (Cohlan, 1954; Giroud and Martinet, 1957).

It would appear that in the pregnant bitch that there would be relatively little likelihood of producing induced congenital malformations from the use of vitamin A at normal therapeutic levels. However, multiple factors are frequently involved in the production of congenital malformations, and lower levels of vitamin A concomitantly with other agents at subthreshhold levels may conceivably result in the production of congenital anomalies.

The use of the pregnant bitch should be expanded in the investigation of experimentally induced congenital malformations. As a result of the work reported here, the canine can be added to the list of animals susceptible to the teratogenic effects of excess vitamin A.

# SUMMARY AND CONCLUSIONS

The existence of a teratogenic effect of hypervitaminosis A in the canine was investigated by daily oral administration of 50,000 to 125,000 I.U. of vitamin A/kg. of body weight to pregnant Beagle bitches. Whelping occurred naturally or Cesarean section was performed. The pups were observed for gross malformations.

Daily doses of vitamin A below 125,000 I.U./kg. of body weight were non-teratogenic with one exception. This was a litter that had minor deformities following a treatment schedule of 50,000 I.U./kg. of body weight from days 17 through 27 of gestation. One pup had accessory auricular structures and two pups had unilateral posterior extensions of the commissure of the lips.

Daily doses of 125,000 I.U. of vitamin A/kg. of body weight had a teratogenic effect when administration included a period covering days 17 through 22 when gestation length was 58 to 62 days. Abnormalities observed were cleft palate, deformed and accessory auricles, tail anomalies, reduced litter size, mummified fetuses, unicornual pregnancy and increased sanguinous amniotic fluid. Thus the canine can be added to the list of animals susceptible to the teratogenic action of hypervitaminosis A.

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# APPENDIX

Table 16. Table of means and standard errors of the means of blood values of rats in experiment I

| | Group | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Hemoglobin (gm./100 ml.) | | | | |
| Day 5 | 14.81±0.20 | 14.76±0.24 | 14.45±0.27 | 15.09±0.26 |
| Day 12 | 14.02±0.20 | 14.48±0.17 | 13.80±0.22 | 14.84±0.33 |
| Day 20 | 13.23±0.24 | 12.56±0.36 | 12.96±0.52 | 14.21±0.37 |
| Methemoglobin (gm./100 ml.) | | | | |
| Day 5 | 0.18±0.04 | 0.95±0.17 | 0.16±0.03 | 1.35±0.35 |
| Day 12 | 0.23±0.03 | 1.66±0.20 | 0.25±0.04 | 1.31±0.36 |
| Day 20 | 0.22±0.04 | 0.98±0.20 | 0.21±0.03 | 1.35±0.36 |
| Packed cell volume (%) | | | | |
| Day 5 | 45.23±0.84 | 43.69±0.54 | 42.32±0.94 | 43.51±0.84 |
| Day 12 | 14.87±0.99 | 42.68±0.58 | 42.09±0.78 | 43.11±0.72 |
| Day 20 | 39.62±0.97 | 39.05±1.00 | 38.39±1.43 | 43.43±0.95 |

Table 17. Table of mean values and standard errors of the means of rats in experiment I

| | Group | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Initial body weight (gm.) | 216±5 | 211±3 | 202±5 | 195±3 |
| Final body weight (gm.) | 285±7 | 264±6 | 247±9 | 216±8 |
| Liver weight (gm./100 gm.) | 3.62±0.09 | 3.41±0.07 | 3.23±0.09 | 3.19±0.08 |
| Thyroid weight (mg./100 gm.) | 4.73±0.20 | 4.17±0.18 | 5.46±0.40 | 5.95±0.38 |
| Pituitary weight (mg./100 gm.) | 3.17±0.13 | 3.34±0.12 | 3.65±0.27 | 3.54±0.15 |
| Adrenal weight (mg./100 gm.) | 19.16±0.89 | 22.33±0.77 | 19.17±1.08 | 23.00±1.18 |
| Number of fetuses per litter | 7.18±0.72 | 7.23±0.88 | 4.58±0.83 | 3.68±0.86 |
| Average weight of fetuses (gm.) | 3.55±0.16 | 3.26±0.15 | 3.65±0.17 | 2.89±0.23 |
| Number of resorptions per litter | 1.82±0.46 | 0.45±0.17 | 0.47±0.19 | 1.45±0.49 |

Table 18. Table of means and standard errors of the means of blood values of rats in experiment II

| | Group | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII |
| Hemoglobin (gm./100 ml.) | | | | | | | | |
| Pre-breeding | 15.47 ±0.37 | 15.17 ±0.31 | 16.98 ±0.47 | 16.45 ±0.32 | -----[a] | ----- | ----- | ----- |
| Day 20 | 12.70 ±0.78 | 12.42 ±0.97 | 13.83 ±0.66 | 13.91 ±0.63 | 11.87 ±0.77 | 12.73 ±0.53 | 13.44 ±0.37 | 12.72 ±0.39 |
| Methemoglobin (gm./100 ml.) | | | | | | | | |
| Pre-breeding | 0.61 ±0.22 | 0.25 ±0.08 | 1.40 ±0.43 | 1.04 ±0.28 | ----- | ----- | ----- | ----- |
| Day 20 | 0.26 ±0.06 | 0.20 ±0.03 | 0.62 ±0.27 | 0.74 ±0.16 | 0.05 ±0.03 | 0.26 ±0.12 | 0.47 ±0.29 | 0.39 ±0.16 |
| Packed cell volume (%) | | | | | | | | |
| Pre-breeding | 39.50 ±1.73 | 38.31 ±1.30 | 44.51 ±1.37 | 44.83 ±1.22 | ----- | ----- | ----- | ----- |
| Day 20 | 36.62 ±0.95 | 38.51 ±1.61 | 37.77 ±1.58 | 39.97 ±1.70 | 35.99 ±2.56 | 37.88 ±1.58 | 41.68 ±1.45 | 39.23 ±1.14 |

[a]Not taken in these groups.

Table 19. Table of mean values and standard errors of the means of rats in experiment II

| | Group | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII |
| Final body weight (gm.) | 341 ±20 | 356 ±16 | 338 ±14 | 328 ±14 | 338 ±12 | 322 ± 9 | 321 ± 8 | 315 ± 6 |
| Liver weight (gm./100 gm.) | 3.49 ±0.09 | 3.35 ±0.10 | 3.34 ±0.15 | 3.67 ±0.19 | 3.41 ±0.12 | 2.82 ±0.28 | 3.19 ±0.11 | 3.17 ±0.11 |
| Thyroid weight (mg./100 gm.) | 5.09 ±0.32 | 3.98 ±0.31 | 4.55 ±0.39 | 4.89 ±0.34 | 4.36 ±0.34 | 6.23 ±0.77 | 5.01 ±0.53 | 5.44 ±0.33 |
| Pituitary weight (mg./100 gm.) | 2.72 ±0.13 | 2.34 ±0.13 | 2.72 ±0.11 | 2.97 ±0.19 | 2.53 ±0.29 | 2.77 ±0.34 | 2.52 ±0.33 | 2.50 ±0.21 |
| Adrenal weight (mg./100 gm.) | 17.81 ±1.39 | 17.05 ±1.14 | 22.21 ±2.28 | 18.57 ±1.21 | 14.95 ±1.45 | 13.04 ±1.43 | 15.83 ±1.06 | 15.44 ±1.37 |
| Number of fetuses per litter | 8.92 ±1.36 | 7.91 ±1.92 | 10.37 ±1.29 | 8.67 ±1.15 | 9.22 ±0.95 | 8.50 ±1.66 | 8.35 ±1.14 | 6.95 ±0.85 |
| Average weight of fetuses (gm.) | 3.89 ±0.19 | 3.67 ±0.07 | 3.68 ±0.34 | 3.61 ±0.22 | 3.43 ±0.14 | 3.46 ±0.20 | 3.35 ±0.15 | 3.50 ±0.16 |
| Number of resorptions per litter | 0.75 ±0.25 | 0.18 ±0.12 | 1.71 ±0.89 | 1.73 ±0.70 | 0.78 ±0.36 | 0.88 ±0.74 | 1.06 ±0.61 | 1.55 ±0.44 |