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Nitrite effects on ascorbic acid metabolism in guinea pigs and rats

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Nitrite effects on ascorbic acid
metabolism in guinea pigs and rats

by

Nieva T. Librojo

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
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INTRODUCTION

The public controversy concerning environmental and dietary causes of cancer has caused many persons to fear dietary sources of carcinogens. Whether these fears have any basis in fact and, if so, what could be done about it is a major public and scientific issue worthy of much research.

Nitrosamines, definite experimental carcinogens in rats, may possibly contribute to the causation of cancer in humans. Several authors have reviewed nitrosamines in this context (Issenberg, 1976; Lijinsky and Epstein, 1970; Magee and Barnes, 1967; Wolff and Wasserman, 1972). The early discovery of dimethylnitrosamines' (DMNA) hepatotoxicity to a number of laboratory animal species (Barnes and Magee, 1954) and production of malignant liver tumors in rats fed diets containing 50 ppm dimethylnitrosamine (Magee and Barnes, 1956) has led to many studies to confirm the validity and to determine the importance of these findings.

The simultaneous presence of nitrite and a nitrosatable amine under conditions such as acid environment in the stomach can lead to nitrosamine formation (Lane and Bailey, 1973; Lijinsky, 1977; Lijinsky et al., 1972). Sources of nitrite in the human diet include preserved meat and nitrate from vegetables and contaminated water (Binkerd and Kolari, 1975; White, 1975; Wolff and Wasserman, 1972). As summarized by Wolff and Wasserman in 1972, reduction of nitrate to nitrite can occur in the microbial environment present in the cattle's rumen, in the infant's stomach which is of lesser acidity under about 4 months of age, in spinach and other high nitrate-containing vegetables cooked or opened from jars of baby food and allowed to stand at room temperature, and in

damp forage materials. Nitrite can be formed from nitrate by microorganisms naturally occurring in the saliva (Tannenbaum et al., 1974).

High nitrate levels can be present in plants which have been harvested from soils that have undergone excessive nitrogen fertilization (Lee et al., 1971). When taken in normal levels, it is not harmful and is a normal constituent of several foods. Excessive nitrate intake, and its reduction to nitrite by microorganisms (Tannenbaum et al., 1974), present a possible health hazard because nitrite can react with certain amines in the stomach to produce nitrosamines (Lane and Bailey, 1973; Lijinsky, 1977; Lijinsky et al., 1972). A Lancet editorial writer in 1977 reviewed epidemiological studies which indicate that, in some countries, high nitrate consumption is associated with human cancer. The deductions of the editor suggest that high nitrate fertilization of crops is the probable cause of nitrate increases in food (Editorial, 1977).

In 1976, Hashimoto et al. added dimethylamine and potassium nitrate to the diet of control and experimental rats. The experimental rats were administered 5 strains of bacteria which form dimethylnitrosamine (DMNA). The stomach, small intestine, and caecum of the experimental rats but not the control rats showed detectable levels of DMNA (Hashimoto et al., 1976).

The addition of nitrite to cured meat and fish serves to arrest the growth of the spore-forming bacterium Clostridium botulinum which can be killed by high heat. During growth, it produces a very potent and highly heat resistant toxin known as botulin. Also, nitrite improves the color and flavor of treated-products (Issenberg, 1976; Wolff and Wasserman, 1972), making them more appetizing for consumers to buy.

Issenberg (1976) summarized the amount of nitrite added to foods; this ranged from 150 to 200 ppm. During processing, most of this disappears which is presumed to be gaseous products, mostly N_2O_3 , NO_2 , and NO (Wolff and Wasserman, 1972). The amount left as nitrite is about 20 ppm or less (Issenberg, 1976).

Since it is obviously impossible to totally eliminate the presence or formation of nitrite in the human body, the risk-benefit method of assessing any hazard should be considered. The levels of nitrite to be used in consumer goods should be the minimum amount that could offer adequate protection. Because different products are exposed to differences in formulation, processing techniques, packaging, and manner of marketing (Christiansen et al., 1973, 1974), this minimum level of nitrite addition that could give the most protection will vary among products. Minimum levels of nitrite usage will lead to lower levels of exposure, but the effect is less than proportional because much of the total nitrite load comes from endogenous synthesis by oral bacteria.

Ascorbic acid has been demonstrated to have a protective effect in blocking the formation of nitrosamines by preventing enzyme activity elevation that follows after nitrite and aminopyrine administration (Kamm et al., 1973). Although intakes of ascorbic acid at levels greater than the recommended dietary allowances seems to offer more advantages than disadvantages as shown in many studies (Archer et al., 1975; Greenblatt, 1973; Kamm et al., 1973; National Academy of Sciences, 1972), some individuals might be sensitive to the toxicity potential of large intakes.

Aside from the protective effect of ascorbic acid against nitrosamine formation, its effectiveness for the partial prevention of methemoglobinemia caused by nitrite was also shown by Stoewsand et al. in 1973. Methemoglobin formation can occur when nitrite comes in contact with hemoglobin (Jukes, 1976). Shearer et al. (1972) have shown that methemoglobin levels of even healthy babies not exposed to diets with high nitrate levels are higher when they are young (31 to 60 days old) than when they are older. This higher methemoglobin level is probably due to the lower stomach acidity of infants at younger age. The risk factors singly taken by Shearer et al. (1972) as contributors to the elevated methemoglobin levels are as follows: high nitrate ingestion (more than 5 mg in 24 hours), illness, location, and contamination of formula by fecal coliform organisms. Minimal exposure to these risk factors with adequate ascorbic acid in the diets of infants and probably older humans may lower the incidence of methemoglobinemia.

With the known effect of nitrite in increasing the ascorbic acid requirement of the guinea pig (Hathcock, 1975), the present studies were made to determine the possible mechanisms of this interaction. It is hoped that the results obtained from these guinea pig studies can be used to project what might be happening in humans, since both species are incapable of making ascorbic acid and therefore dependent on their diets for its supply. The experiment with rats was conducted to study the effects of nitrite on the ascorbic acid metabolism of species that can synthesize ascorbic acid, and do not depend on diet and intestinal absorption for it.

Lastly, these studies may provide more data to help resolve questions of adverse effects of nitrite on health, and the continued use of nitrite as a food additive.

GENERAL REVIEW OF LITERATURE

Functions of Ascorbic Acid

Ascorbic acid is a dietary required vitamin for relatively few species; it seems metabolically indispensable for all mammals and many other animals, but most synthesize it.

Evolution of the biosynthesis of ascorbic acid in animals as reviewed by Chatterjee in 1973 has produced marked differences in ascorbic acid synthesis. Insects, invertebrates, and fishes do not have the ability to synthesize ascorbic acid. In amphibians and reptiles, the site of ascorbic acid synthesis is the kidney. In mammals that are capable of synthesizing ascorbic acid, it is the liver. Guinea pigs, flying mammals, monkey, and man are unable to synthesize ascorbic acid apparently due to genetic deletion of the enzyme gulonolactone oxidase.

Although there is no enzyme system yet described which involves ascorbic acid as a specific coenzyme (White et al., 1973), the versatility of ascorbic acid as a reactant in the body metabolism still is very obvious and well-documented. Ascorbic acid is known to prevent scurvy, a disease which has been a problem in ancient times by men who went on long voyages at sea (Hodges, 1971; Todhunter, 1962).

There are three kinds of doses in which ascorbic acid is administered in the body, as discussed by Hodges (1976). The physiologic dose is 45 mg or less. The pharmacologic dose is 100 to 2,000 mg and is often prescribed with dubious effectiveness for treatment of colds and a variety of other illnesses. The toxic dose ranges from 2,000 to 4,000 mg or more depending on duration. The toxicity manifestations have been

summarized as reproductive failure, interference with tests for glycosuria, reversal of anticoagulant effects, possible induction of nephrolithiasis, inactivation of vitamin B₁₂, and induction of vitamin C-dependent syndrome.

Pregnancy, lactation, environment, and type of diet can affect the physiologic need for ascorbic acid (Hathcock, 1975; Majumder et al., 1975a,b; Mirvish et al., 1972; Nandi et al., 1973; National Academy of Sciences, 1974). The biochemical functions of ascorbic acid include its relationship to cholesterol catabolism (Ginter, 1973; Ginter et al., 1972; Sokoloff et al., 1967), drug and toxicant metabolism (Degkwitz et al., 1973; Mirvish et al., 1972; Pelletier, 1970; Rivers, 1975; Zanoni et al., 1972), mineral metabolism (Gipp et al., 1974; Hill and Starcher, 1965; Majumder et al., 1975b; McCurdy and Dern, 1968), its use as co-substrate for dopamine β -hydroxylase (Friedman and Kaufman, 1966; Levin et al., 1960), and its function in the activation of prolyl hydroxylase (Nutrition Reviews, 1973; Stassen et al., 1973). More controversial functions of ascorbic acid are its relationship to fertility and colds (Briggs, 1973; Clegg and MacDonald, 1975; Hoffer, 1973; Pauling, 1970, 1971).

Dietary and Other Factors Related to Ascorbic Acid

In rats, the site of highest rate of ascorbic acid absorption is the ileum. In guinea pigs, ascorbic acid absorption occurs through the duodenal and proximal intestinal wall (Hornig et al., 1973a). When guinea pigs were given single oral dose of 1-¹⁴C-labeled ascorbic acid, radioactivity was found to be highest in pituitary and adrenal glands,

the central nervous system, the testes, pancreas, and eye lens (Hornig et al., 1974).

Nandi et al. (1973) have found neither beneficial nor toxic effect of large doses of ascorbic acid on growth and maintenance of rats and guinea pigs when these animals were fed nutritionally-balanced fortified wheat diet containing (%): whole grain wheat flour, 63; sucrose, 10; casein, 15; groundnut oil, 5; shark liver oil, 2; USP XVII salt mixture, 4; and AOAC vitamin mixture, 1. Toxicity occurred when a daily intake of 50 mg or more ascorbic acid was given to guinea pigs that were fed unfortified wheat diet. Addition of 15% casein to the unfortified wheat diet was found to alleviate the toxicity of the large doses of ascorbic acid.

In guinea pigs, the protein content of the diet has a great influence on how their tissues retain ascorbic acid. When fed the fortified wheat diet the animals had better ascorbic acid utilization, as shown by their high urinary and tissue ascorbic acid levels. Omission of casein from the fortified wheat diet led to low levels of ascorbic acid in the plasma, adrenal, and urine which were similar to those obtained when only wheat flour was fed (Majumder et al., 1975a). With increased oral ascorbic acid intake, there were increases in the ascorbic acid concentrations in leucocytes and organs until the ascorbic acid requirement of the animal was reached (Keith and Pelletier, 1974).

Supplementary ascorbic acid affects animals that synthesize ascorbic acid by increasing turnover of calcium, phosphate, and the components of organic matrix of the bone (Brown, 1973).

If the results of Nandi's studies on guinea pigs are also applicable to man, then Pauling's (1971) consideration that ascorbic acid can be taken in large amounts without the least danger may be apparently true as long as man consumes an adequate amount of protein in a nutritionally-balanced diet. Excess intake of ascorbic acid, however, may be harmful in areas where the main bulk of the diet consists of cereals (Nandi et al., 1973). However, the problem of formation of urinary stones should be taken into consideration as some individuals have a propensity to form urinary stones when consuming large amounts of ascorbic acid (Atkins et al., 1964).

Male guinea pigs were placed on a scorbutogenic diet for 7 days to deplete tissue ascorbic acid levels. The animals were then given oral dose of 1 mg ascorbic acid daily/100 gram body weight. The feed intake of the experimental animals was limited to 10 grams/animal for the next 8 to 16 days, decreased to 5 grams/animal on days 16 to 19, and then further reduced to 3 grams/animal on days 20 to 24. Control animals were fed ad libitum. On the 24th day, the animals were killed. There was a significant reduction in ascorbic acid concentrations in the spleen, liver, and adrenal glands of the animals fed the restricted diet. There was also a 25 percent body weight loss. It is suggested that lowered metabolism caused by decreased nutrient intake induced decrease in tissue ascorbic acid concentrations (Davies and Hughes, 1977).

Ascorbic acid increases efficiency of iron utilization and also counteracts toxicity resulting from high intakes of iron (Majumder et al., 1975b). Enhancement of iron absorption by ascorbic acid persists with amounts of iron up to 120 mg (aqueous solution) or 105 mg (tablets). It

has been suggested that iron formulations should contain ascorbic acid to permit adequate therapy of iron deficiency anemia without resorting to large iron doses (McCurdy and Dern, 1968).

Studies of Cook and Monsen (1977) indicate that addition of ascorbic acid in the diet would be beneficial in increasing iron absorption and, therefore, ascorbic acid appears to be a practical way of increasing iron utilization and status in regions with high incidence of iron deficiency anemia. Although with ascorbic acid supplementation, iron is still substantially controlled in the intestinal mucosa, the authors warn that ascorbic acid supplementation may be detrimental to patients with idiopathic hemochromatosis, thalassemia major, and sideroblastic anemia. Such persons will have additional increases in already high rates of iron absorption.

Ascorbic acid decreases the absorption and retention of copper (Hill and Starcher, 1965). High intake of copper caused impairment of iron absorption from the gastrointestinal tract and led to iron deficiency. Supplementation of ascorbic acid in the diet decreased the impairment, thus counteracting the detrimental effect of the high copper intake. Two possible mechanisms of the improvement caused by ascorbic acid are: 1) increased absorption and utilization of Fe, and 2) interference with Cu absorption (Gipp et al., 1974).

Results conducted on smokers and nonsmokers with similar characteristics and dietary habits indicate that less vitamin C is effectively available for utilization by smokers. This is evident in the lower vitamin C levels in the blood of smokers as compared to nonsmokers before a load test was given. The smokers had lower excretion or greater

absorption of the test dose given. It is also possible that the smokers may have altered vitamin C metabolism (Pelletier, 1970). It should also be noted that the plasma, leucocyte, and platelet concentrations of ascorbic acid are lowered in women ingesting oral contraceptives. It was shown that it is the estrogenic component of the oral contraceptive that is associated with this decrease in ascorbic acid concentration (Rivers, 1975). Also, ascorbic acid deficiency was observed in 58 percent of the drug addict patients in the National Institute of Mental Health Clinical Research Center in Lexington, Kentucky as shown by their lowered plasma and buffy coat ascorbic acid concentrations (Croft, 1973).

In a study by Burr et al. (1974), there was a higher plasma ascorbic acid and leucocyte ascorbic acid in women than in men. Although this difference may have been caused by the higher fruit consumption by the women, the authors thought this was not entirely responsible for the higher ascorbic acid levels. There might be a physiological difference between the sexes in the retention and metabolism of the vitamin. Lower mean plasma ascorbic acid and leucocyte ascorbic acid were detected in the elderly as compared to the earlier studies conducted on younger persons. The ascorbic acid levels of the elderly seem to have been most affected by the amount of fresh fruits and green vegetables in their diet.

Results obtained by Hornig et al. (1973b) show that administration of ascorbic acid in massive doses to guinea pigs on a long term period has no inducing effect on the ascorbic acid metabolizing enzyme system. These results disagree with the hypothesis that ingestion of ascorbic acid in large doses causes induction of ascorbic acid metabolizing

enzymes as cited in the paper (Hornig et al., 1973b). Also, the authors could not confirm the claim that guinea pigs given supplemental ascorbic acid become scorbutic sooner when fed a deficient diet than do guinea pigs previously fed a normal diet.

Nitrate, Nitrite, Nitrosamines, and Ascorbic Acid

A survey of the circumstances which led to the industrial practice of using nitrate and nitrite as food preservatives was made by Binkerd and Kolari in 1975. Although the origin of the use of nitrates in meat curing is lost in history, the authors are certain that meat preservation with salt came earlier than the intentional use of nitrates by many centuries. Meat preservation seems to have been first practiced in the saline deserts of central Asia and in coastland areas. Desert salts contained nitrates and borax as impurities. However, the reddening effect of nitrates was not mentioned until late Roman times.

Long before Christianity began, "nitre" or saltpeter was obtained in ancient China and India. Ancient people used saltpeter [$\text{Ca}(\text{NO}_3)_2$] which was found as efflorescence on the walls of caves and stables. The authors suggest that due to the nitrate contaminant of the desert and wall salts that were used as preservatives, cured meat with distinctive color and flavor was produced. Phoenicians, Europeans, and Greeks used salt prior to Christianity. The Greeks taught the Romans how to use this preservative.

The use of toasted salt for curing was recommended for hot climates. It could be speculated that this was done to destroy microorganisms or to produce nitrite from nitrate if one assumes that nitrate is a natural

contaminant of the salt. The effect of nitrate on the color production of cured meat was recognized because salting alone (without contaminants) led to loss of the desired meat color.

The first use of nitrite in meat curing is actually unknown. However, classical scientists demonstrated that nitrite and not nitrate was causing the typical color of the meat. In 1923, the Bureau of Animal Industry of the United States Department of Agriculture (USDA) allowed processors to use nitrite in meat under Federal inspection. Several studies were conducted with regard to the use of nitrite in meat curing which led to the formal authorization of the USDA in 1925 to its use in Federally inspected establishments. In 1970, regulations issued under the Meat Inspection Regulations, USDA Consumer and Marketing Service, Federal Register 1970, 35, 15590, permitted addition of sodium or potassium nitrate at 7 pounds to 100 gallons pickle, 3.5 ounces to 100 pounds meat in dry cure, or 2.75 ounces to 100 pounds chopped meat. Sodium or potassium nitrite also was permitted at 2 pounds to 100 gallons pickle at 10 percent level of pump, 1 ounce to 100 pounds meat in dry cure, or 0.25 ounces to 100 pounds chopped meat and/or meat by-product. The finished product should contain not more than 200 ppm nitrite which could come from nitrite and/or nitrate uses as preservative. Studies conducted between 1970 and 1974 showed slight differences in the amounts of nitrite added to various meat products. Even the highest levels used were still consistent with the amounts that Federal regulations permitted (Binkerd and Kolari, 1975).

Normal nitrate intakes are clearly not harmful but upon reduction to nitrite can be a threat to health. Therefore, high nitrate intake under

conditions that are favorable for its conversion to nitrite becomes a hazard (Wolff and Wasserman, 1972). White (1975) has reviewed the relative significance of dietary sources of nitrate and nitrite and states that because the average individual is not viewed as being at special risk, studies should be done on individual situations possibly at greater risk as caused by special diet, age, ethnic background, sex, or area of residence. Since differences exist in the formulation, processing techniques, packaging, and manner of marketing of cured food products, Christiansen et al. (1973, 1974) suggest the amount of nitrite and nitrate necessary to eliminate the hazard brought about by the Clostridium botulinum should be determined separately for each class of product.

Nitrite occurs in the saliva as nitrate is reduced by microorganisms (Tannenbaum et al., 1974, 1976). There was a direct correlation between the nitrate ingested with the amount of nitrite produced. An average increase of 20 ppm nitrite in saliva was formed for every 100 mg nitrate ingested (Spiegelhalder et al., 1976). Tannenbaum et al. (1976) obtained a similar pattern of results. High nitrate led to greater concentration of salivary nitrite although there were large individual differences among the subjects tested.

In adult humans, nitrite poisoning apparently has not been a problem. In infants, however, there were some instances of poisoning from nitrite-containing spinach in baby foods (Wolff and Wasserman, 1972).

Studies conducted on fasting individuals indicate that hypochlorhydric and achlorhydric stomachs provide an environment suitable for nitrate reduction and nitrosamine formation. Hypochlorhydric subjects

have higher concentrations of gastric juice nitrite. Metabolically active bacteria which are capable of converting nitrate to nitrite are active in neutral gastric juice. If nitrosation of amines could occur in the fasting stomach, the level of thiocyanate detected could possibly be favorably used for increased nitrosamine formation (Rudell et al., 1976).

Tumors of the respiratory system developed in male common European hamsters that were subcutaneously administered the volatile carcinogen N-diethyl-nitrosamine (DEN) at dosage of 20 mg/kg body weight once a week for life (Mohr and Hilfrich, 1972). There was a statistically significant increase in the incidence of vascular tumors, mainly in the retroperitoneum of female Swiss mice after a once a week injection (i.p.) of dimethylnitrosamine for 10 weeks (Cardesa et al., 1973). The injection of a single dose of 1.25 mg DEN/kg body weight induced a tubular kidney adenoma in a female Sprague-Dawley rat 108 weeks after treatment (Mohr and Hilfrich, 1972).

Although suggestions have been made to minimize the exposure of humans to nitrosamines and nitrosamine precursors (Lijinsky, 1977; Lijinsky and Epstein, 1970), which may actually be important in lowering the incidence of cancer, the data presented by White (1975) suggest that the amount of nitrite obtained from common food and water supply should not cause alarm to the average individual because the daily nitrite ingestion from food and water amounts to only 4.33 mg/day.

In a review by Jones and Grendon (1975), the environmental factors involved in the origin of cancer and estimation of the possible hazard to man were presented. Jones and Grendon estimated that if the levels

of nitrosamines that caused cancer in laboratory animals were extrapolated to humans, it would probably take 10 to 20 times the human lifespan before tumor could be developed in man. Such dose-lag time extrapolations have been criticized; a dose population probability may be more appropriate. With a dose-population extrapolation, increased dosage of a suspected carcinogen can be tested in a smaller number of animals. Since cancer can only be caused by cancer-causing substances, these higher dosages of administered compound can immediately show us whether a substance is carcinogenic or not. Time and money are saved as compared to the dose-lag time extrapolation experiments which use much larger population of test animals and much longer experimental time before significant results are obtained (Bates, 1977).

Ascorbic acid has been found to have a protective effect on rats that were exposed to nitrite and aminopyrine (Kamm et al., 1973). Ascorbic acid caused important nitrite losses when added to endogenous or normally used concentrations in meat. Under nitrogen, the production of nitric oxide was lower than the loss of nitrite which indicates that there are probably semistable nitroso intermediates formed that do not release nitrite on Griess analysis. There may be some reactions undergone by the intermediates other than releasing free NO or nitrite (Fox and Nicholas, 1974). The effectiveness of ascorbic acid in preventing the formation of the mutagenic material produced in fish treated with high levels of nitrite was shown by Marquardt et al. (1977).

Much research has shown that reaction of nitrite with secondary and tertiary amino compound can lead to formation of N-nitroso compounds. The reaction can occur in vivo as well as in nitrite-containing food.

In vivo, the stomach provides the most favorable environment for the reaction. It is possible that formation of N-nitroso compounds, especially nitrosamides, could pass into the colon and cause tumor induction in that organ (Lijinsky, 1977).

The presumptive toxicity of nitrite relates to its oxidation of hemoglobin and its reaction with amines or amides to form carcinogenic nitroso compounds under normally encountered situations (Wolff and Wasserman, 1972). Many drugs contain secondary amines, alkyl urea, or carbamate groups. If such drugs are orally administered at a time when high nitrite containing foods are in the stomach, possible hazard to health might occur (Mirvish et al., 1972).

Ayanaba et al. (1973) suggested that nitrosamines may be present in water that is used for drinking or bathing and thus may be responsible for most of the hazard associated with nitrates and nitrites in water. Edible crops may assimilate nitrosamines from the soil. It is also possible that after leaching or soil erosion, these compounds may move into waterways. These possible ways in which man could be exposed to nitrosamines could lead to increased nitrosamines in his body systems. The authors were able to show that dimethylnitrosamines can be formed by the activities of microorganisms in sewage and soil.

The N-nitrosamines, dimethyl-N-nitrosamines, and N-nitrosopyrrolidine have been detected in food products (Fong and Chan, 1976, 1977; Panalaks et al., 1973, 1974; White et al., 1974).

Mirvish et al. (1972) suggested that drugs containing secondary amine, alkyl-urea, or N-alkyl-carbamate groups with a substance that preferentially reacts with and destroys any nitrite group occurring in

the stomach might reduce the possible hazard which carcinogenic N-nitroso compounds formation might produce. Data were presented showing that ascorbic acid could be used for this purpose.

Although ascorbate increased the nitrosation of dimethylamine under some conditions, the chemical results gathered in the in vitro studies of Mirvish et al. (1972) seem to support the suggestion that ascorbate should be combined with potentially nitrosatable drugs before drug administration. It may be possible that this combination of ascorbate with certain drugs might lessen the production of carcinogenic N-nitroso compounds.

At a pH 4.0 in an aerobic condition, it was shown that N-nitrosomorpholine formation was completely inhibited when the ascorbate/nitrite molar concentration ratio was 0.5. All nitrite was not consumed at lower concentrations of ascorbate, thus allowing some nitrosomorpholine formation. The same pattern of observation occurred at pH 3.0 and 3.5 (Archer et al., 1975).

In a study conducted by Greenblatt (1973), a single gavage of 17.3 μ moles 4-dimethyl aminopyrine and 58 μ moles sodium nitrite (NaNO_2) were administered to mice. This dose produced acute centrilobular necrosis of the liver in 48 hours through the toxicity of the DMNA formed. Ascorbic acid was added to the gavage mixture in different concentrations. Results showed that hepatic necrosis could be completely prevented when the concentration of ascorbic acid was twice the molar concentration of NaNO_2 . This protective effect of ascorbic acid was not complete when its concentration was equimolar to that of NaNO_2 .

There seems to be an important role of ascorbic acid in the normal attachment of ribosomes to endoplasmic reticulum to produce polyribosomes.

It is possible that the degranulation of the rough endoplasmic reticulum which is associated with the action of some carcinogens may be a symptom of the ascorbic acid's rapid depletion (Edgar, 1974). Furthermore, Kakar and Wilson (1976) conclude that there is preferential accumulation of ascorbic acid in neoplastic tissues which is probably the reason why leucocyte and plasma ascorbic acid values of individuals with cancer are lower than those obtained in normal subjects.

Even with supplemental dosage of ascorbic acid, Feller et al. (1975) were not able to detect measurable ascorbic acid in parotid and whole saliva with the use of a quantitative procedure that involved paper chromatographic separation and gas-liquid chromatographic analyses. They found measurable levels of ascorbic acid with the use of 2,4-DNP colorimetric method. The differences in these results may be due to interfering substances which give false positives in the 2,4-DNP assays. Whole blood ascorbic acid determinations have been endorsed as a potentially better means of assessing ascorbic acid body store due to the fact that ascorbic acid contributions of all the components of the blood are determined when the whole blood is used for analysis (Pearson, 1967; Roe and Kuether, 1943).

Nitrites, Ascorbic Acid, and Methemoglobinemia

An example of nitrite toxicity is the methemoglobinemia produced by the oxidation of hemoglobin by nitrite (Wolff and Wasserman, 1972). Rodkey (1976) demonstrated the stoichiometry of the reaction for the conversion of oxyhemoglobin to methemoglobin by nitrite. For every mole of oxyhemoglobin iron changed to methemoglobin, 1.5 moles of nitrite is

oxidized to nitrate. One mole of proton is consumed in this reaction and no oxygen is liberated.

Concentration of methemoglobin above 70 percent would lead to asphyxia. The reaction is reversible at lower methemoglobin levels (Phillips, 1971), but these lower levels might cause decreased oxygen transport capability.

The lesser stomach acidity in infants make them more susceptible to methemoglobinemia than adult humans (Shearer et al., 1972; Wolff and Wasserman, 1972). Stoewsand et al. (1973) demonstrated the protective effect of ascorbic acid against nitrite-induced methemoglobinemia. When high levels of ascorbic acid were combined with methionine, a synergistic effect in reducing nitrite-induced methemoglobin was produced. The fact that it easy to obtain foods that contain substantial amounts of ascorbic acid may be important in preventing methemoglobinemia in humans.

RESEARCH REPORT

ABSTRACT

These studies were designed to further study the known nitrite-caused increased ascorbic acid requirement in guinea pigs.

In a completely randomized design with a 2 x 2 factorial arrangement of treatments, guinea pigs fed ascorbic acid (0 or 200 mg/kg) and NaNO_2 (0 or 300 mg/kg) for 21 days were injected with 1- ^{14}C labeled ascorbic acid.

Dietary nitrite significantly lowered the whole-blood concentrations of ^{14}C of ascorbic acid-treated guinea pigs after 3 hours, but significantly increased adrenal contents of ^{14}C at both levels of dietary ascorbic acid. The amount of expired $^{14}\text{CO}_2$ was not affected by dietary nitrite or ascorbic acid.

Nitrite effects on ascorbic acid degradation and clearance were determined in guinea pigs. High vitamin C, but not dietary nitrite, increased the urinary oxalate excretion. As expected, the total ascorbic acid blood levels were increased by ascorbic acid, but lowered by nitrite addition in the diet. Adrenal ascorbic acid concentrations were not significantly affected by nitrite.

Rats showed a decrease in blood ascorbic acid levels with increased dietary nitrite. Gulonate:NADP oxidoreductase activities were lowered by nitrite.

INTRODUCTORY REVIEW

Nitrite is widely used as a curing agent for meats. It is found in human saliva at levels of about 6 to 10 ppm (Tannenbaum et al., 1974). Under physiological conditions, nitrate is easily reduced to nitrite. It has been estimated that the average person in the United States derives four-fifths of the nitrate intake from vegetables (White, 1975). A potentially troublesome source of nitrite for livestock is stored rainwater containing decaying organic matter (Counter et al., 1975; Gibson, 1975).

The conversion of hemoglobin to methemoglobin can be brought about by nitrite, thereby making less oxygen available to the tissues (Sleight et al., 1972). Ascorbic acid has been shown also to partly prevent methemoglobin formation (Stoewsand et al., 1973). In vivo and in vitro studies have shown that, under appropriate conditions, nitrite can interact with secondary and tertiary amines to produce N-nitroso compounds (Lijinsky and Taylor, 1977).

Carcinogenic nitroso compounds may have an important role in cancer etiology (Lijinsky and Taylor, 1977; Magee and Barnes, 1967). The studies of Akin and Wasserman (1975) show a protective effect of ascorbic acid against carcinogenesis in animals concurrently receiving nitrosamine precursors. In vitro ascorbic acid can block the nitrosation of several secondary and tertiary amines (Mirvish et al., 1972). The use of Na ascorbate in vacuum-packed wieners does not appreciably alter the effect of NaNO_2 in inhibiting Clostridium botulinum toxin formation (Bowen et al., 1974); therefore, continuation of use of nitrite as a curing agent in the presence of ascorbate is likely.

The present studies were conducted to determine the possible mechanisms involved in the nitrite effect (Hathcock, 1975) in increasing the ascorbic acid requirement of guinea pigs and to determine whether metabolically similar effects occur in rats. Data will be discussed on dietary nitrite effects on blood levels of vitamin C and gulonate NADP oxidoreductase, an enzyme collected from liver cytoplasmic supernatant of rats fed different levels of dietary nitrite.

MATERIALS AND METHODS

Experiment 1: Twelve male albino guinea pigs with weights between 200 and 300 grams were purchased from Biolab.¹ They were individually housed in stainless-steel wire cages. Feed and water were given ad libitum during the 3-week treatment period. Except for ascorbic acid, the basal diet (Table 1) used meets the nutrient requirement of guinea pigs (National Academy of Sciences, 1972). The animals were fed the basal diet with ascorbic acid and NaNO₂ added in a 2 x 2 factorial arrangement of treatments at dietary concentrations of 0 or 200 mg/kg and 0 or 300 mg/kg, respectively.

Table 1. Ascorbic acid-deficient basal diet

Component	Percent
Casein	25.0
L-Arginine·HCl	1.0
Cellulose	15.0
Starch	46.0
Vitamin premix ^a	1.0
Mineral premix ^b	7.0
Corn oil	5.0

^aVitamin premix provided: (mg/kg diet) choline chloride, 2500; folic acid, 20; niacin, 50; calcium pantothenate, 40; pyridoxine·HCl, 20; riboflavin, 20; thiamin, 20; vitamin B₁₂, 2; vitamin A acetate, 15; and vitamin D₃, (IU/kg diet) 2000.

^bMineral premix provided: (g/kg diet) Williams-Briggs salt mix, 40; K₂CO₃, 20; CaCO₃, 6; and MgO, 4.

¹Biolab, St. Paul, Minnesota.

At the end of the third week, each guinea pig was injected with $1\text{-}^{14}\text{C}$ labeled ascorbic acid, $10\ \mu\text{Ci/kg}$. The $^{14}\text{CO}_2$ expired during the next 3 hours was trapped in NaOH (1N). The animals were lightly anesthetized with ether, and blood samples were obtained by cardiac puncture with needle and heparinized syringe. The animals then were killed with excess ether, and the adrenal glands were removed and immediately weighed.

The blood samples, adrenal glands, and NaOH solution were assayed for ^{14}C radioactivity by liquid scintillation counting by using channels ratio quench correction (Herbert, 1965). Factorial analysis of variance (Steel and Torrie, 1960) was used for statistical evaluations of whole-blood dpm/ml , CO_2 dpm/kg/hr , and adrenal dpm/g .

Experiment 2: With the same experimental design as in experiment 1, 28 male albino guinea pigs weighing between 140 and 225 grams were randomly assigned to treatments with 7 animals per treatment. The feed¹ was in crushed pellet form rather than the powder feed used in experiment 1.

At the end of the third week, the animals were fasted for 16 hours; then urine was collected. A modification of Fraser and Campbell's method for the determination of oxalate in urine was used (Drewes, 1974). The principle involves precipitation of oxalate from acidified urine by an excess CaCl_2 at pH 4.5 in the presence of a known amount of added oxalate. The washed calcium oxalate precipitate is then dissolved in H_2SO_4 . Calcium then is determined by atomic absorption. Blood samples and

¹Purchased from Teklad Standard Diets, Winfield, Iowa.

adrenal glands were collected and assayed for total, reduced, and dehydroascorbic acid (Roe and Kuether, 1943).

One part of whole blood was added to 3 parts of 6 percent trichloroacetic acid (v/v) in a test tube, shaken, allowed to stand for 5 minutes, and then centrifuged for 10 minutes at 2,500 rpm. Supernatant was poured off and acid-washed Norit¹ was added to the supernatant for the total ascorbic acid determination but was omitted in the dehydroascorbic acid (DHA) determination.

The Norit-treated supernatant was filtered through Whatman No. 42 filter paper. One drop of thiourea and 1 ml of 2,4 dinitrophenylhydrazine solution were added to 4 ml of filtrate or supernatant in test tubes (for total ascorbic acid or DHA determination). The test tubes were incubated for 3 hours at 37°C and then chilled in crushed ice. Five ml of 85 percent sulfuric acid (H₂SO₄) were added to each tube. The solutions were mixed by twirling, allowed to stand, and then read against a reagent blank at 515 nm. Concentrations were obtained by use of a standard curve with ascorbic acid ranging from 0 to 60 µg per tube. The reduced ascorbic acid was obtained by subtracting the value of DHA from the total ascorbic acid.

For the determination of the ascorbic acid levels in the adrenal glands, the same procedure was followed except that 4 percent trichloroacetic acid was used to treat the minced adrenal gland. Care was taken

¹ Acid-washed Norit is prepared by using USP grade of activated carbon. Add 1 liter of 10 percent hydrochloric acid (HCl) to 200 g of activated carbon, heat to boiling, and filter with suction. Stir the cake of Norit with 1 liter of water and filter. Further addition of water to the cake of Norit is not necessary if the washings give a negative or very faint test for ferric ions. Dry the cake in an oven overnight at 100-120°C.

to insure that the volume of the TCA used for the given weights of adrenal glands was sufficient to give total ascorbic acid and dehydroascorbic acid values that could be read from the standard curve.

Statistical evaluations were made by factorial analysis of variance with the method of unweighted means (Bancroft, 1968).

Experiment 3: Male weanling Wistar rats (3 weeks old) were obtained from the stock colony in the Department of Food and Nutrition at Iowa State University, Ames, Iowa. A completely randomized design with 3 x 5 factorial arrangement of treatments and time was used. Five rats were randomly assigned to treatments and individual stainless-steel wire cages and given water and feed ad libitum. The animals were fed a basal diet as shown in Table 2 for a 2-week adjustment period.

Table 2. Steenbock diet XVII (males), modified 5/6/76

Ingredient	Percent
Corn meal	45.6
Skim milk powder	10.3
CaCO ₃ + trace elements	0.4
NaCl (iodized salt)	0.4
Yeast	8.6
Casein (raw)	7.0
Alfalfa meal	1.7
Wheat germ	10.8
Linseed meal	11.6
Corn oil	3.5
Vitamin D ₃ in corn oil ^a	0.1

^aCrystalline vitamin D₃ (cholecalciferol) diluted with corn oil to give 2,000 IU or 50 mcg vitamin D₃ per kg diet. Solution A: Dilute 500 mg crystalline vitamin D₃ with 399.5 g corn oil. Solution B: Dilute 20 g of solution A with 480 g corn oil. Use 1 g of solution B per kg diet.

At the end of the second week, which was considered as zero day, the animals were fed the basal diet with 0, 300, or 900 mg NaNO_2 /kg feed with 5 rats per treatment. They were killed by cervical dislocation 1, 11, 22, 33, or 44 days after being fed their respective diets. Blood for total ascorbic acid determination (Roe and Kuether, 1943) was collected by cardiac puncture with needle and heparinized syringe. The liver was immediately removed for gulonate NADP oxidoreductase assays of the cytoplasmic supernatant.

The enzyme was analyzed by using the spectrophotometric method of ul Hassan and Lehninger (1956) as used by Stubbs and Haufrect (1968) with modifications. The cytoplasmic supernatant solution was prepared by adding 2.5 volumes of 0.15 M KCl per gram of liver. The sample was then homogenized and placed in plastic tube for refrigerated (4°C) centrifugation at $3,000 \times g$. Cytoplasmic supernatant, 10 μl , or equivalent buffer was introduced to 6 ml of the assay mixture which contained 3 mM sodium D-glucuronate, 10 mM nicotinamide, 0.1 mM NADPH, and 50 mM tris buffer (pH 7.6). Except for the omission of NADPH, the blank solutions were the same. The samples were read in a spectrophotometer at 340 nm before and after incubation in air at 37°C for 20 minutes.

Protein was determined by using the biuret method (Gornall et al., 1949). Biuret reagent was prepared by adding 300 ml of 10 percent NaOH (w/v) to a solution consisting of 1.5 g cupric sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and 6 g of sodium potassium tartrate tetrahydrate ($\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$). The cytoplasmic supernatant solution was diluted with water to give values that could be read with the bovine serum albumin standard (0-10 mg/ml). Biuret reagent, 4.0 ml, was added to 1.0 ml of

diluted cytoplasmic supernatant or standard solution, mixed, allowed to stand for 30 minutes at room temperature, and read against a blank at 540 nm. Values of samples (mg/ml diluted solution) were obtained by comparing with the curve for the bovine serum albumin standard.

RESULTS AND DISCUSSION

Guinea pigs were used in the first two experiments because, like humans, they cannot synthesize ascorbic acid. The third experiment used rats to determine the effect of nitrite addition on the ascorbic acid metabolism of test animals that are capable of vitamin C synthesis.

Experiment 1: There were no gross observable differences in the physical appearance of animals fed the ascorbic acid-deficient diet containing 0 or 300 mg NaNO_2 /kg basal diet. The animals generally were inactive, but became nervously active when approached for observation, feeding, or watering. All ascorbic acid-deficient animals lost weight; some had very distinct alopecia, and some showed bleeding gums by the end of the three-week experiment.

Guinea pigs fed the ascorbic acid-supplemented diet with or without NaNO_2 gained weight. They generally were active yet were not nervous when approached.

Rats also can metabolize parenterally injected 1- ^{14}C ascorbic acid to $^{14}\text{CO}_2$. Furthermore, there is a further loss of radioactive material by urinary excretion of either metabolites or unaltered ascorbic acid (Burns et al., 1951, 1954).

Table 3 shows the dpm of ^{14}C obtained from whole blood and adrenal glands of guinea pigs given different dietary treatment combinations after intraperitoneal injection with 1- ^{14}C vitamin C.

Table 3. Dpm of ^{14}C obtained after intraperitoneal injection of 1- ^{14}C -ascorbic acid in guinea pigs^{1,2}

Compounds added in mg/kg		Adrenal dpm	Whole-blood dpm	$^{14}\text{CO}_2$ dpm
Ascorbic acid	NaNO_2	g ($\times 10^5$)	ml ($\times 10^6$)	kg/hr ($\times 10^4$)
0	0	0.68 ^a	1.29 ^a	16.91 ^a
200	0	1.09 ^b	2.45 ^b	5.67 ^a
0	300	1.03 ^b	0.79 ^a	3.16 ^a
200	300	1.33 ^c	1.39 ^a	11.49 ^a

¹Numbers without same superscript are significantly different ($P \leq 0.05$).

²Each entry is average of 3 animals.

The ^{14}C contents of adrenal glands from ascorbic acid-deficient and supplemented guinea pigs were significantly increased by dietary nitrite. With and without dietary nitrite, ascorbic acid addition led to significant increases in ^{14}C contents of adrenal glands.

With dietary ascorbic acid, addition of nitrite significantly decreased the ^{14}C contents of whole blood. Without dietary ascorbic acid, nitrite addition did not significantly decrease the ^{14}C levels of whole blood. The lack of significant effect seems reasonable biologically inasmuch as the animals were ascorbic acid deficient, and blood ascorbic acid levels were already low, even without nitrite effect.

As expected, ascorbic acid addition led to increased concentrations of ^{14}C in whole blood from guinea pigs not treated with nitrite. In nitrite-treated guinea pigs, addition of ascorbic acid to the diet did not significantly increase the whole-blood ^{14}C . There was a substantial and perhaps real increase in this value, but the effect of ascorbic acid was not significant in this experiment and the increase, if any, seems to be smaller than in the animals without nitrite.

The results of these studies did not show significant effects of nitrite or ascorbic acid on the amount of expired $^{14}\text{CO}_2$. Labeled ascorbic acid degradation or clearance may have been taking place via different routes in the body.

Ascorbic acid or nitrite might have had marked effects on the amount of expired $^{14}\text{CO}_2$ if larger doses of the compounds had been used. The purpose in these studies was to test amounts of ascorbic acid that would be sufficient for maintenance of normal growth and normal blood concentration. The level of nitrite was the same as used previously in this laboratory (Hathcock, 1975).

The 1- ^{14}C atom of the vitamin C molecule may have been metabolized or translocated as part of the intact ascorbic acid to other tissues. Because it is not known whether the ^{14}C remained as part of the vitamin C molecule or as part of an ascorbic acid metabolite, a follow-up experiment was done.

Experiment 2: Experiments to determine nitrite effects on ascorbic acid degradation and clearance were conducted by using the same dietary treatment design as in experiment 1, but with increased numbers of test animals.

Physical appearance and activity patterns of the guinea pigs were similar to those in experiment 1. Table 4 shows the blood ascorbic acid levels (total, dehydro, and reduced) and Table 5 gives the total ascorbic acid present in the adrenals as affected by the diet combinations.

Table 4. Total ascorbic acid, dehydroascorbic acid, and reduced ascorbic acid in blood samples of guinea pigs as affected by sodium nitrite and ascorbic acid addition in the diet

NaNO ₂ (mg/kg)	Dietary ascorbic acid (mg/kg)								
	0			200			Mean		
	Total ascorbic acid	Dehydroascorbic acid	Reduced ascorbic acid	Total ascorbic acid	Dehydroascorbic acid	Reduced ascorbic acid	Total ascorbic acid	Dehydroascorbic acid	Reduced ascorbic acid
	mg ascorbic acid/100 ml blood								
0	0.52 (5) ^a	0.77 (6)	0.64	0.36 (5)	0.51 (6)	0.44	0.16 (5)	0.26 (6)	0.21
300	0.43 (5)	0.71 (7)	0.57 ^{**}	0.34 (5)	0.47 (7)	0.40 ^{NS}	0.09 (5)	0.24 (7)	0.16 ^{NS}
Mean	0.48	0.74 ^{**}		0.35	0.49 ^{**}		0.12	0.25 ^{**}	

^aNumbers in parentheses indicate the numbers of observations.

^{**}Highly significant differences between means ($P < 0.01$).

^{NS}Not significantly different.

Table 5. Total ascorbic acid in adrenals of guinea pigs as affected by sodium nitrite and ascorbic acid addition in the diet

NaNO ₂ (mg/kg)	Dietary ascorbic acid		Mean
	0	200	
	Mg total ascorbic acid/g adrenal		
0	0.36(6) ^a	0.50(7)	0.43
300	0.28(6)	0.57(7)	0.42 ^{NS}
Mean	0.32	0.53 ^{**}	

^aNumbers in parentheses indicate the numbers of observations.

^{NS}Not significantly different.

^{**}Highly significant differences between means ($P \leq 0.01$).

As expected, there was a significant increase in the total ascorbic acid, dehydroascorbic acid, and reduced ascorbic acid blood levels upon addition of ascorbic acid in the diet. Nitrite addition decreased blood total ascorbic acid significantly at each dietary level of ascorbic acid. Nitrite had no effects on ratios of DHA to reduced ascorbate in whole blood at either dietary level of ascorbate. The effect of nitrite on the total blood ascorbic acid levels is in agreement with the lowered whole-blood 1-¹⁴C ascorbic acid in experiment 1.

The total adrenal ascorbic acid was increased with increased ascorbic acid intake. Nitrite addition, however, did not produce any significant change in the total ascorbic acid levels of the adrenals. This result is in apparent conflict with the increased ¹⁴C dpm in the adrenals with nitrite treatment in experiment 1. It is possible that adrenal ¹⁴C in experiment 1 may not have been part of any ascorbic acid molecule, but instead have been in some metabolite that does not assay as ascorbic acid.

It is well-known that vitamin C can have an effect in increasing urinary oxalate (Atkins et al., 1964), although individual ability to produce oxalate as induced by vitamin C addition has been reported to vary (Briggs et al., 1973; Hagler and Herman, 1973; Roth and Breitenfield, 1977). The findings of Briggs (1976) indicate some type of enzyme-induction mechanism, which shows high sensitivity to excess ascorbic acid in affected individuals. Although the increase in oxalate after large doses of vitamin C is small in most subjects (Lamden and Chrystowski, 1954), Briggs (1976) suggests that it is likely that enzymes of the ascorbate oxalate pathways are of low induction.

Dietary nitrite and ascorbic acid effects on the amounts of urinary oxalic acid dihydrate per 24 hours for every 100 g body weight of guinea pigs are shown in Table 6. High vitamin C intake, but not dietary nitrite, increased the urinary oxalate excretion. These results show that the nitrite effect on ascorbic acid nutrition is not through increased conversion of ascorbic acid to oxalate.

Table 6. Effects of dietary NaNO_2 and ascorbic acid on urinary oxalic acid dihydrate excretion by guinea pigs

NaNO ₂ (mg/kg)	Dietary ascorbic acid (mg/kg)		
	0	200	Mean
Mg oxalic acid dihydrate/24 hr/100 g			
0	3.98(4) ^a	6.65(6)	5.32
300	1.47(4)	6.72(7)	4.10 ^{NS}
Mean	2.72	6.68*	

^aNumbers in parentheses represent number of observations.

^{NS}Not significantly different.

*Significant differences between means ($P \leq 0.05$).

Determination was made of the urinary total vitamin C, DHA (dehydroascorbic acid), and reduced ascorbic acid. Nitrite had no effects on urinary excretion of these forms of ascorbic acid.

Experiment 3: The nitrite treatments made no obvious differences in physical appearance and activity patterns of the rats. Table 7 shows the amounts of NADPH consumed by the enzyme gulonate NADP oxidoreductase obtained in the liver cytoplasmic supernatant per minute per mg of protein of male rats fed control and two levels of sodium nitrite and killed at different times.

Table 7. NADPH utilized by gulonate NADP oxidoreductase obtained from liver cytoplasmic supernatant of male rats fed different levels of sodium nitrite before they were killed^{1,2}

Days fed <u>ad libitum</u> preceding killing	Dietary NaNO ₂ (mg/kg)		
	0	300	900
	Nanomoles/min/mg protein		
1	27.8 ^a	25.8 ^b	23.1 ^c
11	29.2 ^a	30.1 ^a	28.9 ^a
22	30.4 ^a	30.5 ^a	29.6 ^a
33	30.1 ^a	30.2 ^a	29.2 ^a
44	29.6 ^a	30.3 ^a	23.5 ^b
Mean	29.4 ^a	29.4 ^a	26.9 ^b

¹Statistical comparisons are between values with different nitrite levels at each time, not between times at each nitrite level; numbers without same superscript are significantly different ($P \leq 0.05$).

²Each entry is average of 5 animals.

As shown in Table 7, (day 1) sodium nitrite, 900 mg/kg led to a significant decrease ($P \leq 0.05$) in the NADPH oxidation by the gulonate NADP oxidoreductase as compared with those fed 0 and 300 mg/kg sodium nitrite. There also was a significant decrease in activity in rats fed sodium

nitrite, 300 mg/kg, compared with the control rats ($P \leq 0.05$). Because the levels of NADPH consumption reflect the amount of gulonate NADP oxidoreductase in the liver cytoplasmic supernatant, these results suggest that dietary nitrite probably depresses in vivo activity of the enzyme. More dietary nitrite caused more extensive depression of the enzyme activity, which suggests decreased vitamin C synthesis.

At days 11, 22, and 33, there were no significant differences observed in the NADPH consumption of the enzyme, indicating that there seems to be a period of adaptation of the rat liver enzymes when exposed to nitrite.

On the 44th day, the NADPH consumptions of the livers of the rats fed the 900 mg/kg diet were significantly lower ($P \leq 0.05$) than those at 0 and 300 mg/kg diet, but no significant difference was observed between the NADPH consumption of animals on the 0 and 300 mg sodium nitrite/kg treatment. It seems that 900 mg/kg dietary nitrite initially depresses the activity, then the activity returns to normal, and is finally depressed again. This could reflect an initial sensitivity, compensation, and a cumulative effect.

Figure 1 shows these changes in enzyme activity with time. The marked changes observed are the low levels of NADPH consumption at day 1, followed by a period of adaptation, and finally by decline in activity in the high-nitrite group. There were no significant differences between enzyme activity of the control and the 300 mg sodium nitrite/kg-treated rats.

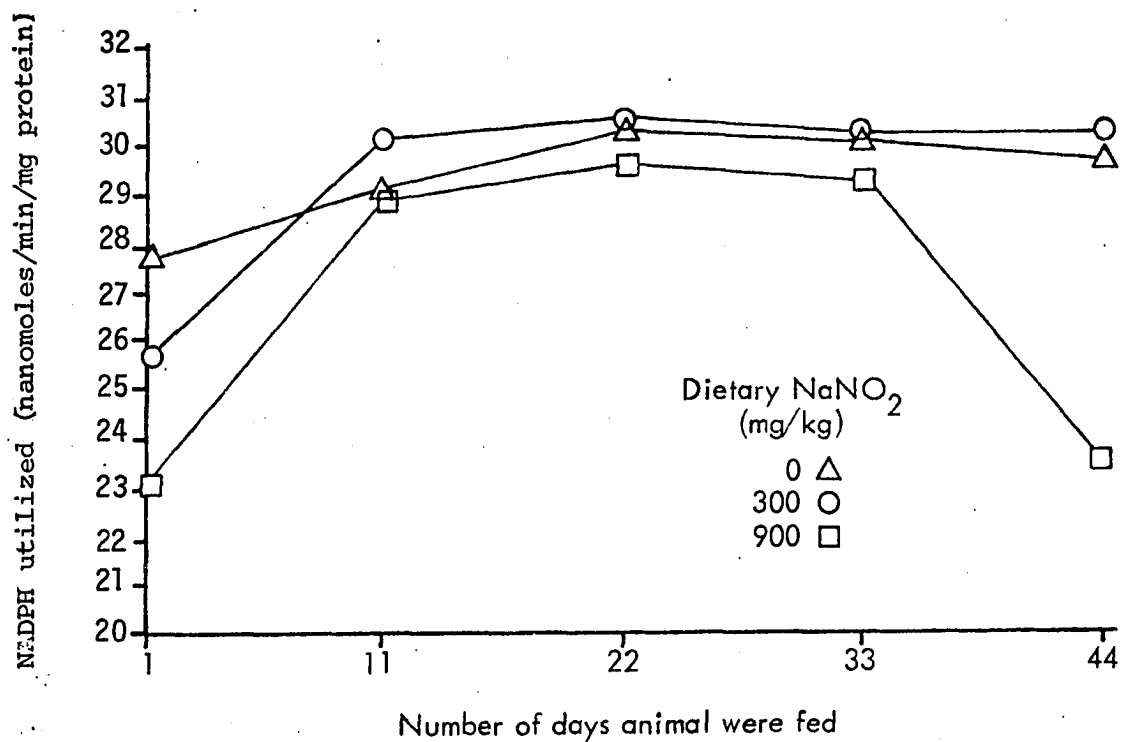


Figure 1. Gulonate:NADP oxidoreductase activities in cytoplasmic supernatant from livers of rats treated with nitrite

The total ascorbic acid blood decrease at day 44, 900 mg sodium nitrite/kg feed, is consistent with the decreased gulonate NADP oxidoreductase activity for the same day and diet supplementation as shown in Table 8. Except for one point on day 11 with 300 mg sodium nitrite/kg feed, all enzyme activity values shown in Figure 1 are correlated ($r = 0.77$) with the blood ascorbic acid values in Figure 2.

Table 8. Total ascorbic acid blood levels of male rats fed different levels of sodium nitrite before being killed^{1,2}

Days fed preceding killing	Dietary NaNO ₂ (mg/kg)		
	0	300	900
	Mg total ascorbic acid/deciliter of blood		
1	0.57(4) ^a	0.61(4) ^a	0.53(3) ^a
11	0.91(3) ^a	0.53(3) ^b	0.95(3) ^a
22	1.04(3) ^a	0.98(3) ^a	0.96(3) ^a
33	1.09(5) ^a	1.01(4) ^{ab}	0.88(5) ^b
44	1.04(3) ^a	0.95(4) ^a	0.54(3) ^b
Mean	0.93 ^a	0.81 ^b	0.78 ^b

¹Statistical comparisons are between values with different nitrite levels at each time, not between times of each nitrite level; numbers without same superscript are significantly different ($P \leq 0.05$).

²The numbers in parentheses are the number of samples collected.

There was a significant decrease in the total blood ascorbic acid by dietary nitrite addition (300 mg or 900 mg/kg feed vs. 0 mg/kg feed, $P \leq 0.05$), although no significant difference was observed in total blood ascorbic acid levels of rats fed the two levels of nitrite.

Earlier dietary studies on rats by Stubbs and Griffin (1973a,b) did not show any diminishing effect of protein deficiency or fasting on gulonate NADP oxidoreductase activity. The present study was done to determine whether activity of this enzyme is affected by dietary nitrite

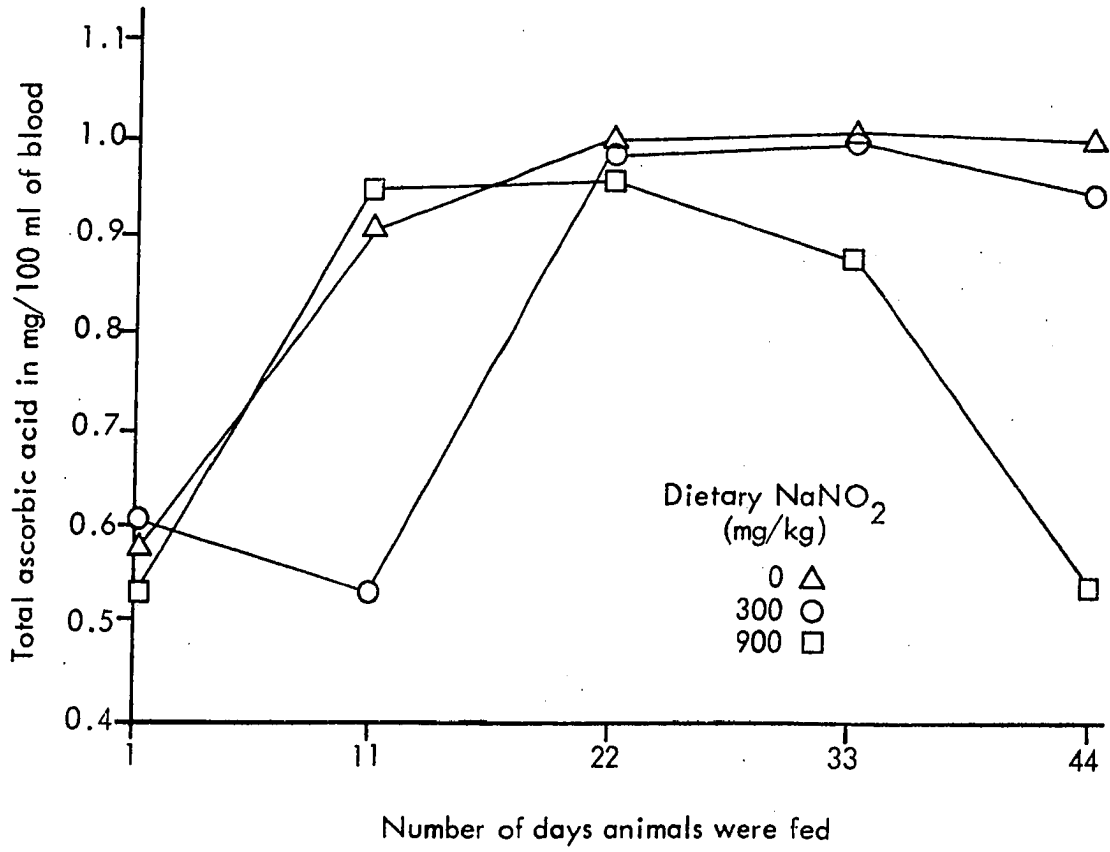


Figure 2. Effects of dietary sodium nitrite on blood ascorbic acid levels in rats

in rats fed ad libitum. The depression of this enzyme activity by nitrite suggests a decreased rate of ascorbic acid synthesis in the nitrite-treated rats. This effect could not account for the effects of nitrite on ascorbic acid in guinea pigs because this species does not synthesize this vitamin. These results provide no direct evidence on possible effects of nitrite on ascorbate degradation rate in rats. The decrease in the activity of gulonate:NADP oxidoreductase and in blood ascorbate concentrations in nitrite-treated rats were quantitatively similar. This suggests the effects of nitrite in the rat could be limited to inhibition of this enzyme, rather than including stimulation of degradation, as in guinea pigs.

Although in vitro studies could have been done with guinea pig small intestine to determine whether nitrite interferes with ascorbic acid absorption, such a study was not made because the experiment with rats fed ascorbic acid-free diets bypassed the intestinal absorption phase of ascorbic acid metabolism. The similar effect of nitrite in decreasing blood ascorbate levels in these two species suggests that the effect in the guinea pig is not due to malabsorption of ascorbic acid. The depression in the total ascorbic acid blood levels in guinea pigs by dietary nitrite does not demonstrate whether nitrite interferes with ascorbic acid absorption or increases metabolism or excretion.

No ascorbic acid supplement was given to the rats because these animals can synthesize their own ascorbic acid from the diet. The decrease produced by nitrite in total ascorbic acid blood levels and activity of gulonate NADP oxidoreductase, one of the enzymes involved

in the biosynthesis of ascorbic acid in rats, suggests that the changes caused by dietary nitrite treatment of the guinea pig may be produced by a similar mechanism.

CONCLUSIONS

Dietary ascorbic acid, with or without nitrite addition, led to increased ^{14}C in whole blood and adrenal glands of guinea pigs injected with $1\text{-}^{14}\text{C}$ ascorbic acid. Conversely, whole-blood ^{14}C was depressed and adrenal ^{14}C was increased by nitrite at each level of dietary ascorbic acid. There were no significant effects of nitrite or ascorbic acid on the amount of $^{14}\text{CO}_2$ expired after $1\text{-}^{14}\text{C}$ -ascorbic acid injection.

In guinea pigs, addition of ascorbic acid led to significant increases in the total ascorbic acid, dehydroascorbic acid, and reduced ascorbic acid concentration of whole blood. Dietary nitrite decreased blood total ascorbic acid significantly at each dietary level of ascorbic acid. Nitrite had no effects on ratios of DHA to reduced ascorbate in the whole blood at either dietary level of ascorbate. The decreasing effect of nitrite on the total blood ascorbic acid levels is in agreement with the lowered whole blood $1\text{-}^{14}\text{C}$ ascorbic acid. Increased ascorbic acid intake led to increased total adrenal ascorbic acid but nitrite addition did not produce any significant change in the total ascorbic acid levels of the adrenals. Urinary oxalate excretion is increased by dietary ascorbic acid, but not with dietary nitrite, indicating that the effect of nitrite on ascorbic acid nutrition is not through increased conversion of ascorbic acid to oxalate.

In rats, there was a significant decrease in the total blood ascorbic acid with dietary nitrite treatment. Activity values of gulonate NADP oxidoreductase seems similarly decreased and therefore compatible with the blood ascorbic acid levels.

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