

**Trace Substances
and
Health**

**A HANDBOOK
Part II**

edited by

Paul M. Newberne

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Paul M. Newberne

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MARCEL DEKKER, INC.

New York and Basel

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MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Library of Congress Catalog Card Number: 75-25167
ISBN: 0-8247-1850-x

Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

Preface

The first Handbook of this series dealt with natural and man-made agents which become a part of our bodies and which may have beneficial or detrimental effects on our health and longevity. This series of two volumes continues in that vein and takes the reader further into the realm of substances heretofore considered of little consequence (plant toxins, nitrates, nitrites) or those which have created such public confusion (nitrosamines, air-borne particulates) that further considerations of available information are in order.

For example, nitrates and particularly nitrites have been a part of our effective arsenal of preservatives and disinfectants, useful in preserving and extending or enhancing meat products for more than a century. It has been only in recent years that their safety has been questioned, first as components of nitrosamines and more recently, as potential carcinogens in their own right. There is no evidence to date, despite the documented carcinogenic potency of nitrosamines in many animal species, that these substances have caused cancer in human populations. Even more remote is the evidence for nitrites as potential carcinogenic agents in human or animal populations. Nevertheless, these substances have created a great deal of concern in the minds of scientists as well as the lay public. The evidence as it exists at the point of publication of these volumes is presented in clear form for nitrates, nitrites, and nitrosamines. These subjects are covered by Paul M. Newberne and Adrienne E. Rogers, both of whom have conducted extensive research in the respective areas for more than a decade.

Plant toxins have been a scourge of both man and animals but also have provided benefits as components of medicinals for the many centuries that mankind has existed. Plant toxins have been used to eliminate enemies, the unneeded and other segments of our human populations. Conversely, plant toxins have also served useful purposes when used in controlled exposure. The potential for anticarcinogenic effects of plant toxins has now been recognized and this is a very active area of intensive research described in considerable detail by A. Robin

Mattocks who has spent much of his productive career examining questions about the pyrrolizidine alkaloids and mechanisms by which they produce their effects.

Those who reside on the coast of the United States as well as those who visit coastline areas where seafood is abundant recognize the real and potential problems of shellfish poisoning and other aspects of seafood toxicity. It is rare indeed to have a clear and lucid résumé of the subject compiled in a chapter prepared by two of the recognized international experts in the field, John C. Wekell and John Liston. They have presented the subject matter in a manner easily understood by both the scientists and the layman.

These volumes continue along lines found to be useful as they appeared in the Handbook, Part I. Résumés, details, and literature references provide scientists and lay public alike with the needed information or an entry into a more extensive literature on the specific subject. These volumes will be of particular interest to those involved in food science research as well as to researchers in biological sciences in general, public health officials, physicians, veterinarians, and the lay public. Since the first volume was published we have learned by trial and error as a society that decisions about matters which impinge on the health of broad segments of the populations can no longer be left in the hands of scientists, politicians, and regulatory agencies; the public in general wants input into the decision-making process and a rational approach will depend on up-to-date- accurate information. These volumes will help in providing such information in a balanced, interpretative manner.

Paul M. Newberne

Contents

Preface	iii
Chapter 1	
NITRATES AND NITRITES IN FOODS AND IN BIOLOGICAL SYSTEMS	1
Paul M. Newberne	
I Introduction	1
II Sources of Nitrate and Nitrite	3
III The Toxic Effects of Nitrate and Nitrite on Man and Animals	15
IV The Use of Nitrate and Nitrite in Meat Preservation: A Historical Note	19
V Characteristics of Meat Cured with Nitrite	21
VI Botulism	24
VII Nitrite As a Direct Cause of Cancer	26
VIII The MIT Nitrite Study	30
IX Animal Studies and Relation to Human Conditions	34
X Risk Assessment	37
XI Summary	39
References	41
Chapter 2	
NITROSAMINES	47
Adrianne E. Rogers	
I Introduction	47
II Chemical Structure, Formation, and Metabolism of Nitrosamines	50

III	Toxicity of Nitrosamines	54
IV	Mutagenicity of Nitrosamines	56
V	Carcinogenicity of Nitrosamines	56
VI	Modification of Nitrosamine Carcinogenesis	75
VII	Summary	75
	Abbreviations	76
	References	76

Chapter 3

PLANT TOXINS	81
A. Robin Mattocks	
I Introduction	81
II General Nature of the Problem	82
III Toxins Originating in Plants	85
IV Detection and Analysis	100
V Summary	101
References	101

Chapter 4

SEAFOOD BIOTOXICANTS	111
John C. Wekell and John Liston	
I Introduction	111
II Intoxications	116
III Comments	143
References	147
AUTHOR INDEX	157
SUBJECT INDEX	173
Contributors	176

Nitrates and Nitrites in Foods and in Biological Systems

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I. Introduction	1
II. Sources of Nitrate and Nitrite	3
III. The Toxic Effects of Nitrate and Nitrite on Man and Animals	15
IV. The Use of Nitrate and Nitrite in Meat Preservation: A Historical Note	19
V. Characteristics of Meat Cured with Nitrite	21
A. Chemical Reactions	21
B. Color	22
C. Flavor	23
D. Oxidation Rancidity	23
VI. Botulism	24
VII. Nitrite As a Direct Cause of Cancer	26
VIII. The MIT Nitrite Study	30
IX. Animal Studies and Relation to Human Conditions	34
X. Risk Assessment	37
A. Probable Fate of Nitrite As a Food Additive	38
XI. Summary	39
References	41

I. INTRODUCTION

This chapter examines nitrate and nitrite in our food supply, air, and water, and how these chemicals may be produced in biological systems. Additional discussions center on the real or potential health problems

which may accompany exposure to nitrate, nitrite, and, briefly, to nitrosamines. It is both the external sources of nitrate and nitrite and the internal synthesis and recycling of these chemicals which are important to the health and welfare of man and animals. The subject of nitrates and nitrites has been one of continuing controversy during the past decade and shows few signs of abating.

Nitrate and nitrite and the controversy surrounding these chemicals relate to the central issue of a safe food supply which has been of great concern to the public in recent years. It has become quite popular to demand a food supply that is absolutely safe, free of harmful chemicals, and sufficiently nutritious to support optimal health. Nutritious food is essential to life but it is comprised of a mixture of thousands of chemicals, any of which can be harmful and even fatal if consumed in sufficient amounts. Vitamins A and D, the amino acid methionine, and trace elements copper and zinc are good examples of nutrients that are essential to life but if consumed in excess and for significant periods of time can be injurious and even fatal. Human beings cannot live without these and other essential nutrients but in excess they are toxic.

In considering the food supply and food additives such as nitrate and nitrite, it is essential to distinguish between toxicity and actual hazard. A food chemical may or may not be hazardous depending upon the amount that is ingested and on other factors. The great concern of the public in demanding absolute safety in foods is mistaken because the generally accepted concept that foods can be free of chemicals is false. All foods consist exclusively of chemicals and all substances are toxic in some degree and therefore are potentially harmful. The concern evidenced by the public in recent years is misdirected because the more important dangers associated with food consumption are of a different nature in our society. It is clear now that in the United States and in many Western countries the chief hazards from foods result from excessive consumption, particularly of those foods rich in fats and in highly refined carbohydrates. These types of foods are associated with obesity, hypertension, cardiovascular disease, dental caries, and directly or indirectly with some forms of cancer.

Even if an individual escapes the hazards of overindulgence of ordinary foods, moderate amounts of some foods may still be hazardous because of some of the chemical substances they contain. These may be added deliberately or they may find their way into the food chain as unintentional additives. The latter is usually a consequence of human activities or they may derive from intrinsic factors which are a part of the plant or animal material as it was synthesized in nature. On the other hand, the human population needs to understand that there is little reason to fear a particular food chemical simply because an overwhelming excess may be harmful. Mankind has throughout history, and for the foreseeable future will continue to ingest chemicals that would be toxic if consumed in excessive amounts. This is a normal situation and

it is impossible for living organisms to avoid taking toxic substances into their body since all substances as noted above are toxic if taken in sufficiently excessive amounts. Relevant considerations center on (1) how much of the substance is consumed, (2) the effect of this amount in a dose-response relationship, and (3) the benefit derived from the material eaten in relation to the potential risk involved.

Further to these considerations, public health authorities should be informed about the possible dangers related to widespread, long-standing practices previously regarded as safe. We are constantly learning that some of the natural compounds considered safe for decades or even centuries are indeed sources of potential or real hazard because of their chronic toxicity or cancer-producing qualities. Nitrate and nitrite fall into this category and continued surveillance and regulatory action based on the best knowledge available is essential to the insurance of public health. Nitrate and nitrite as chemicals are discussed in this chapter; the additional concern of the combination of nitrite with amines to produce nitrosamines are considered in detail in Chap. 2.

II. SOURCES OF NITRATE AND NITRITE

Nitrate salts are widely distributed in water, soil, and plants; in addition, nitrogen that occurs in other forms along with nitrates, when acted upon by living systems, form nitrites. Nitrites continuously form and break down in nature, the process by which nitrogen in the atmosphere is converted to a useful form and ultimately returned to the atmosphere again in what is referred to as the nitrogen cycle. The increasing concentration of nitrates in water results primarily from pollution by organic wastes and from fertilization of crops by chemical fertilizers which have a high nitrogen content. For example, more than 11 million tons of nitrogen fertilizers are added to the soil in the United States each year [1]. In areas where nitrogen-containing fertilizers are used extensively, both the soil and the water may contain large amounts of nitrates and nitrites. It appears, however, that the amount of nitrate and nitrite added to the environment by human action is considerably less than the amount that is present naturally.

Vegetables commonly consumed by human populations contain significant quantities of nitrates as shown in Table 1. From these data it is clear that many of the commonly consumed staple vegetables contain considerable amounts of nitrate which, when taken into the body, is converted to nitrite. Celery, radishes, beets, and leafy vegetables including lettuce, cabbage, spinach, and broccoli are especially rich sources. The average levels of nitrate range from 13 ppm in some samples of dry beans to as much as 3000 ppm or more in beets [2]. Moreover, 15,000 ppm of nitrate has been found in radishes [3]. Nitrites as such do not usually occur in large amounts in food or water. However, when foods are rich in nitrate and are acted upon by bacteria there is

TABLE 1. Estimated Average Daily Per Capita Ingestion of Nitrate and Nitrite by U.S. Residents

Source	Nitrate-nitrogen		Nitrite-nitrogen	
	mg	% of total	mg	% of total
Vegetables	19.4	86.0	0.06	1.8
Fruits, juices	0.3	1.3	0.00	0.0
Milk and products	0.05	0.2	0.00	0.0
Bread	0.5	2.2	0.01	0.3
Water	0.2	0.9	0.00	0.0
Cured meats	2.1	9.3	0.72	21.1
Saliva	6.8	---	2.62	76.8
Total	22.55	99.9	3.41	100.0

Source: Adapted with permission from W. J. White, Jr., Journal of Agricultural Food Chemistry, 24:202, 1976. Copyright 1976 by the American Chemical Society.

continual increase in the amount of nitrite present. Thus any food containing natural or added nitrate will eventually contain nitrite.

If we are to understand the factors that influence the nitrate-nitrite content of foods, consideration must be given to the sources and movement of nitrogen and its compounds in the environment. It is generally assumed that the origin of nitrogen used in biological reactions is primarily atmospheric N_2 . It is very likely, however, that a major portion of the earth's nitrogen is contained in its fundamental rocks [4]. While much of the nitrogen contained in rocks may be available, this source, because of the total content, may provide a larger proportion of the nitrate in waters than is believed to be the case [5].

Exactly how nitrate-nitrite compounds are formed in the atmosphere is not clearly understood but it is generally believed that nitrous oxide (N_2O) and nitric oxide (NO) are biological in origin [6]. The most abundant oxide of nitrogen in the atmosphere is N_2O , where the concentrations range from 500 to 1200 mg/m^2 [7]. Fossil fuels which are on the increase in use around the world generates principally NO , and this is subsequently oxidized to NO_2 . The photolytic cycle of the nitrogen oxides has been recently described [8].

It has been pointed out that a significant quantity of the nitrogen compounds found in natural water comes from precipitation [9]. The amount of ammonia delivered to earth in rain or snow is twice that of nitrate. However, a 17-year study in Canada indicated that nitrate plus nitrite accounted for about 35% of the total nitrogen in rain and snow, with a total nitrogen amount reaching the land each year of about 2 tons/mile² of nitrate equivalent or about 7 lb/acre. This is

in agreement with levels reported in European observations. The amount of nitrite in rain and snow is very likely small but the few studies that have been made do not permit accurate assessment. It appears, however, that only about 1 to 5% of the amount of nitrate occurs as nitrite.

There are a number of complex and close interrelationships affecting the content of nitrate-nitrite in soil and water. In addition to the nitrogen compounds delivered by precipitation, as mentioned earlier, there is also the direct addition of fertilizer which contains ammonia, urea, and nitrates; the fixation of nitrogen from the atmosphere by microorganisms and legumes; the microbial decomposition of nitrogenous compounds in plants and man's sewerage and wastes; and the geochemical leaching of nitrate into groundwater [10-12].

It would appear that the amount of nitrogen biologically converted to ammonia and ultimately into nitrates is quite large compared to that derived from direct precipitation (200 lb of nitrogen per acre per year, compared to 7 lb per acre per year). Nitrogen fixation is achieved by an enzyme system, nitrogenase. This is distributed among a number of apparently unrelated organisms or plant microorganisms (symbiont). It has been described by Hardy and Burns [13] as a multiprotein complex containing nonheme iron, labile sulfide and molybdenum, a complex that catalyzes the reduction of N_2 and a number of nitriles and alkynes, the generation of hydrogen, and the concomitant hydrolysis of Adenosine 5'-triphosphate (ATP).

There is some degree of specificity of microbial oxidation of soil ammonia derived from decayed processes or from added ammonia. *Nitrosomonas* and related genera convert ammonia to nitrite, while *Nitrobacter* and related genera oxidize nitrite to nitrate, a two-stage process accomplished by the soil microorganisms [14,15]. It is now known that a number of plants readily absorb leachable nitrate ion and then reduce it to nitrite by nitrate reductase enzymes [16]. These enzymes are molybdo-iron-flavoproteins and organisms such as *Pseudomonas*, *Proteus*, *Escherichia coli*, among others, carry out such reactions. Some plants do not use absorbed nitrate, and in such cases nitrate is converted to ammonia in the roots.

Methemoglobinemia in human infants is one of the most striking toxic effects of consuming excess nitrate which is converted to nitrite and combines with hemoglobin to form methemoglobin. In the case of infants this usually results from consuming bottle formulae made with water from wells containing high concentrations of nitrate. This was recognized and described by Comly in 1945. Following the first report in the medical literature, more than 2000 cases of methemoglobinemia have been recorded as a result of consuming water containing nitrates [17,18]. A more recent report in 1964 by Holscher and Natzschka [19] of cyanosis in infants from spinach resulted in the renewed interest in the nitrate-nitrite content of plants in soils and of the various factors which influence concentrations of these compounds. This subject will be dealt with more in detail later in this chapter.

Factors which are known to affect the nitrate content of plants include the species, variety, stage of maturity, and the part of the plant. Environmental factors which result in an increase in nitrate include high temperature, drought, cloudiness or shading, deficiencies in certain nutrients such as calcium and phosphorus, excessive nitrogen from legume residues and fertilizer. In addition, insect damage and herbicides contribute to the nitrate content of plants.

Nitrate-nitrite poisoning in livestock has been recorded in the veterinary literature for many decades [20]. One of the more recent serious episodes in the United States occurred during the summers of 1953 and 1954 in the Midwest; Missouri was particularly affected [21]. This occurred as the result of a complex of environmental factors: severe drought, extreme heat, heavy fertilization of crops, particularly corn, and the use of weed-killing chemicals. In attempts to salvage the corn that had been stunted as a result of drought, farmers allowed cattle to graze the fields, or they harvested the cornstalks to store in the silo as silage. As a result of the high nitrate content of the cornstalks, large numbers of cattle were lost from methemoglobinemia. In addition, a number of human lives were also lost when the silos were being filled with the high nitrate-containing corn; farmers descended into the silos to aid in distributing the ensilage and were overcome by the oxides of nitrogen from the ensilage; some of them succumbed as a result.

Comparing the levels of nitrates in vegetables in 1964 with an earlier study made in 1907 indicated that there was a similar pattern of nitrates in vegetables in spite of the shift from organic fertilizer such as manure in the 1907 case to more extensive use of chemical fertilizers in recent years [22]. As pointed out earlier, certain vegetables such as spinach, beets, radishes, and lettuce tend to have a higher concentration of nitrate, but a wide variability is common between and within species of vegetables. There is substantiating evidence that the use of chemical fertilizers and increasing nitrogen content over the past several decades has had an influence on the concentration of nitrates in the soil and water. It is interesting, however, that the magnitude of the increase is not necessarily related to the level of fertilization, as shown by Brown and Smith [23]. These investigators concluded that the effects were more noticeable in the early maturing species of vegetables, but it also seems clear that fertilization rates greater than 50 lb of nitrogen per acre are necessary to produce a significant effect on nitrate-nitrogen in plants.

A number of other factors are of consequence to the content of nitrate-nitrite in substances. For example, fresh spinach normally contains little if any nitrite; however, within the first few days of storage at room temperature there is a loss of nitrate and an increase in nitrite [24]. For the next few days both nitrate and nitrite decrease. Refrigeration delays but does not prevent the production of nitrite. When the spinach was frozen, however, nitrite-nitrogen

concentration only reached the level of about 1 ppm up to 5 months under freezing conditions. These observations clearly indicate the effects of storage on the levels of nitrate and nitrite in spinach. It is also established that no nitrite production appears to take place during commercial canning processes. It thus appears that the conversion of nitrate to nitrite or ingestion is a principal potential hazard, but conversion in the gastrointestinal tract in humans has not been fully documented. There are some data available from recent studies relative to endogenous synthesis of nitrate/nitrite which will be alluded to later on in this chapter.

Two other groups of investigators have studied the accumulation of nitrate in vegetables with particular attention to spinach and beets [25,26]. The rate of application of nitrogen to the soil under the conditions of these studies ranged from 0 to 400 lb of nitrogen per acre. More than one type of nitrogen carrier was used as well as three types of spinach and one type of beets. Nitrate concentration in the vegetables did not appear to be linearly related to the amount of nitrogen applied to the soil; interestingly, the response was proportionately greater at the lower levels.

It has been pointed out by Lee [27] that although the general trend toward higher levels in certain vegetables is clear, the actual variations are so large that the effect of various agricultural fertilization practices could only be evaluated by specially designed experimentation. The opinion has been expressed by many that attempts to control nitrate by monitoring food plant levels would present insurmountable problems. Furthermore, although there is little in the literature on the potential for breeding low nitrate strains of vegetables, it is evident [28,29] that varieties do vary greatly in their nitrogen-containing properties. The reader is referred to many literature references [30]35] regarding nitrate levels in plants and the complex interrelationships which influence them, including soil chemistry, microbiology, and geochemical factors.

In regard to vegetable and plant sources of nitrate, it is of interest to note that although dairy cattle may consume forage containing considerable quantities of nitrate, little of the nitrate appears in the milk. Cured meats supply a relatively small amount of nitrate, a part of which is added in the curing process and a part of which develops from the oxidation of some of the nitrite used in curing. As referred to later, a significant amount of nitrate nitrogen is secreted in saliva and swallowed in that form, thus constituting a considerable source of nitrate/nitrite.

Regardless of how nitrates enter the body, once inside, some proportion of those consumed are converted to nitrites. Bacteria in the mouth are responsible for the conversion of part of the nitrate in human saliva to nitrite, perhaps on the order of 10 mg/day. Bacteria in other parts of the digestive system are also thought to produce nitrites from nitrates [36]. Actually very little is known about the

metabolism of nitrate and/or the proportion of the nitrate that enters the body that is converted to nitrite. Studies with human subjects have shown wide variation among individuals and although the percentage of ingested nitrate converted to nitrite is not known, it is likely to be significant. For example, Tannenbaum and collaborators have shown that bacterial reduction of nitrate in saliva may be the major mode of nitrite formation [37]. Other studies have shown that vegetables and vegetable juices rich in nitrate could result in levels of salivary nitrite reaching hundreds of milligrams per liter by way of a process of recirculation of unmetabolized nitrate though the blood by way of the salivary glands.

In further studies designed to assess the extent of nitrate intake in the population through measurements of salivary and urinary nitrate and nitrite, balance studies were conducted in human subjects [36]. Six Caucasian males ranging in age from 68 to 72 years and weighing from 57 to 105 kg participated in the nitrate balance studies. All were judged to be healthy according to physical examination and clinical and laboratory tests. The subjects were studied as inpatients at the Clinical Research Center at the Massachusetts Institute of Technology in Cambridge, Massachusetts. After having been fed a diet of their own choosing for at least a week, the subjects were then given a protein-free diet which contained 6 mg of nitrogen per kilogram of body weight per day for 10 days. Following this they were given a diet containing 0.8 g of egg protein per kilogram of body weight per day. The latter diet was supplied in a liquid formula for 10 days, and urine and dietary components analyzed for nitrate and nitrite concentrations.

Nitrate is absorbed from the proximal small intestine and cleared from the plasma by the kidney [38,39]. It was thus expected that the subject would reach equilibrium rapidly and that the urinary excretion would reach a steady state, the concentration of which would represent some fraction of the nitrate that was consumed. Instead of reaching a steady state it was found that there were significant fluctuations in urinary nitrate on a day-to-day basis on both the 0.8 g and on the protein-free diets. Table 2 shows a comparison of total nitrate intake and average output for both dietary regimens. The differences for each individual on both of the diets were so great that the only explanation was some significant source of nitrate other than the diet. Urinary nitrate is apparently unrelated to the level of protein in the diet and for this reason it is unlikely that muscle catabolism was involved in these studies. This then leaves the intestine as the most likely site of endogenous nitrate/nitrite synthesis. It has been considered by many that intestinal microflora play an important role in the synthesis, degradation, and flux of nitrate-nitrite in the biological systems, but this is yet to be established.

Tannenbaum et al. further found in healthy Caucasian males from 25 to 40 years of age, wide variations in the concentrations of nitrite and nitrate in fecal samples. Table 3 illustrates these findings in

TABLE 2. Total Daily Dietary Nitrate Intake Compared with Average Daily Urinary Nitrate Excretion

Subject	Diet	Intake ^a ($\mu\text{mol NaNO}_3$)	Excretion (μmol)
1	Protein-free	72	884
	0.8 g/kg	140	551
2	Protein-free	54	1660
	0.8g/kg	86	1300
3	Protein-free	72	1800
	0.8 g/kg	155	1830
4	Protein-free	61	1360
	0.8 g/kg	117	1350
5	Protein-free	85	655
	0.8 g/kg	140	1230
6	Protein-free	60	1230
	0.8 g/kg	117	814

^aDaily intake was constant over the experimental period.

Source: Ref. 36. S. R. Tannenbaum, D. Feh, V. R. Young, P. D. Land, and W. R. Bruce. *Science* 200:1487, 30 June 1978. Copyright 1978 by the American Association for the Advancement of Science.

TABLE 3. Nitrate and Nitrite Levels in Human Feces and Intestinal Contents.

Subject	Sample	NO ($\mu\text{mol}^3/\text{kg}$)	NO ($\mu\text{mol}^2/\text{kg}$)
A	Fecal	174	77
B	Fecal	124	112
C	Fecal	N.D. ^a	407
D	Fecal	71	106
E	Fecal	N.D.	186
F	Fecal	34	48
G	Fecal	487	339
H	Fecal	N.D.	425
I	Ileostomy	N.D.	1510
J	Ileostomy	N.D.	1540
K	Ileostomy	N.D.	350
L	Ileostomy	N.D.	880
M	Ileostomy	N.D.	530
N	Ileostomy	N.D.	760

^aN.D., not detectable (<2 $\mu\text{mol}/\text{kg}$).

Source: Ref. 36. S. R. Tannenbaum, D. Fett, V. R. Young, P. D. Land, and W. R. Bruce. *Science* 200: 1487, 30 June 1978. Copyright 1978 by the American Association for the Advancement of Science.

addition to the findings of nitrate-nitrite content in the effluent from ileostomies from six Caucasian males 25 to 30 years of age. These sample analyses revealed similar fluctuations in nitrite but, interestingly, no detectable nitrate. The absence of nitrate in the ileostomy samples along with such high nitrite concentrations suggest that nitrite is synthesized from a more reduced nitrogen compound.

It would seem that a logical explanation for these results would be that heterotrophic nitrification of ammonia or organic nitrogen compounds takes place in the upper portion of the intestine where the environment is aerobic. As material passes through the intestine the environment becomes more anaerobic which may lead to the accumulation of nitrate as well as nitrite. Some of these compounds are undoubtedly utilized by other fecal microorganisms while some may be absorbed through the intestinal wall as either nitrate or nitrite. These data imply that human exposure to nitrite is probably greater than previously recognized.

The nitrite that is absorbed is rapidly destroyed in the blood but the nitrate is recycled into saliva by way of the salivary glands and in the stomach by way of the parietal glands [49]. It seems feasible that in this manner intestinal nitrification may contribute to the etiology of cancer in humans, but this is only speculative. In fact, it is only speculative as well that microflora contribute significantly to the recycling of nitrate and nitrite in humans and other animals, but it appears to be a logical explanation. Furthermore, the evidence is accumulating to suggest that nitrites other than those ingested from the air, water, and food make considerable contributions to the total body exposure to nitrite. These additional sources of nitrite located within the normal healthy body is therefore less amenable to control than are those exogenous sources that occur primarily in the food and water supplies. Nevertheless, it is essential to look at the dietary and environmental sources of nitrate and nitrite in conjunction with the healthy body's natural production and endogenous synthesis of these compounds in order to develop a complete assessment of exposure.

Total exposure to nitrate and nitrite, while not easily or accurately measured, is of sufficient interest to justify extensive well-designed experimental studies. It would seem that one must deal separately with nitrite which is introduced into or formed in the mouth and then enters the stomach, and with nitrite which is formed in the intestine, converted to nitrate in the blood, and which ultimately contributes to the amount of nitrite that is formed in saliva. The preformed nitrite entering the mouth must be balanced against the nitrite which is formed in saliva as the result of the reduction of nitrate. Certain aspects of the nitrite that is formed in the saliva in the gastric compartment and in the intestine will be considered according to some of the proposals put forth by Tannenbaum [45].

Salivary nitrite has been known for more than 100 years. The nitrite in saliva arises from nitrate by bacterial reduction; saliva