The Sodium/Iodide Symporter, Nitrate, and Dental Caries:
A Perspective from a Review of the Literature

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Abstract: Nitrates have been used as an antimicrobial agent in foods for hundreds of years. As a result there is extensive literature on their mode of activity in the food sciences and other such diverse fields as gastroenterology, the pulmonary sciences, and cancer research but minimal research exists in the dental sciences. This manuscript will describe how nitrate present in green leafy vegetables is absorbed and concentrated in saliva. Through the intervention of facultative anaerobes in the oral cavity inert nitrate is converted to nitrite which through acidification becomes a potent nitrosating agent. The sodium/iodide symporter is responsible for the transport of nitrate from serum to saliva and agents that inhibit this process, goitrogens, will be shown to have an impact on caries rates. It is the nitrosation of critical sulfhydryl groups in glycolytic enzymes and carrier molecules that is responsible for the suppression of acid production and therefore the reduced cariogenicity of lactic acid bacteria. The difficulty in identifying nitrites behavior as an anticariogenic agent may stem from its cyclic nature due to its reliance on dietary intake.

The sodium/iodide symporter,NIS, is a glycoprotein comprised of 618 amino acids.(1,2,3). It has the ability to concentrate iodide in thyroid cells, thyrocytes, against its electrochemical gradient by a factor of from 20 to 40 with respect to plasma, noted as T/P or T/S, thyroid to serum.(4,5). Two Na^+ ions are transported with each anion and the energy released by the translocation down its electrochemical gradient is coupled to the transport of iodide uphill against its electochemical and concentration gradient. The energy needed for this process is derived from Na^+/K^+ ATPase.(1,5,6). The iodide is incorporated into the thyroid hormones T3, triiodotyrosine, and T4, thyroxine, in a process termed organification. The NIS is present not only in the thyroid but also a number of extrathyroidal tissues. These include the salivary glands, gastric mucosa, lactating mammary glands, chroid plexus, placenta, and kidney tubules.(1,4,7). All these tissues exhibit a number of similarities which include, 1) the ability to concentrate iodide by a factor of 20 to 40, 2) antagonistic inhibition of this concentrating mechanism by anions such as thiocyanate, SCN^-, perchlorate, $ClO4^-$, nitrate, $NO3^-$, and selenocyanate, $SeCN^-$, and 3) concentration of a number of anions other than iodide.(1). TSH, thyroid stimulating hormone, produced in the pituitary gland serves as a regulator by stimulating NIS gene and protein activity in thyrocytes which increases iodide uptake by the thyroid gland.(3). An important difference between the thyroid and salivary glands is that TSH has no impact on the activity of the salivary glands.(4,7,8,9). The function of the thyroid, whether it is hypothyroid, euthyroid, or hyperthyroid, also appears to have no influence on the salivary NIS in its anion transporting capabilities.(10). In addition, non-thyroidal tissues do not have the ability to produce T3 or T4. Also the salivary glands can concentrate thiocyanate by a factor of 20 unlike the thyroid where it is oxidized.(1,11). In spite of these differences the close association of the

salivary and thyroid NIS is indicated by the fact that an autosomal recessive genetic defect, Congenital Iodide Transporter Defect, ITD, expresses itself concurrently in both glands.(2,3,7,12). The character of the NIS in the thyroid and salivary glands has been claimed to be very similar if not identical.

   Evidence for the association between the Na/I Symporter and Dental Caries involving cationic amino acid transport.

   A study was conducted by B.C. VanWuyckhuyse et al. analyzing the association between free amino acids with caries experience. Adults are grouped as being caries susceptible,(CS), or caries free,(CF). There was a positive relationship between a reduced caries rate and the cationic amino acids arginine and lysine in parotid saliva. The p value for histidine is just beyond statistical significance.

Table 1 (Modified from table 2, ref.13)

asp	0.109	gln	0.215	ala	0.555	met	0.052	leu	0.238
glu	0.009	his	0.009	arg	**0.0006**	trp	0.126	lys	**0.0009**
asn	0.482	gly	0.800	tyr	0.169	phe	0.164	hyp	0.043
ser	0.757	thr	0.673	val	0.448	ile	0.646	pro	0.798

CS adults vs. CF adults, Bonferroni constant, p/20

 These are the three cationic, positively charged, amino acids with magnitude of positive charge being arginine, pKr =12.5, lysine, pKr =10.5, and histidine, pKr = 6.0. The reason given in the study for the cariostatic effect of arginine is that bacteria can produce NH_4^+ by way of the arginine deiminase system, ADS, and that this increases the oral pH in caries free individuals.(13). In rebuke of this hypothesis Calandra and Fosdick determined that the separate addition of a wide range of amino acids including arginine, lysine, and histidine did not increase the pH in a mixture with saliva and glucose at physiological concentrations.(14). The justification for lysine activity is that it has a cariostatic effect when delivered systemically.(15,16).
   In a study by L. Vranic et al. significantly lower levels of arginine were found in children with caries and there was a complete lack of histidine when compared with a control group. Concentrations of lysine were not examined.(17). In an additional study by W. Giebel et al. only amino acids with non-polar residues occurred in all samples,(n=17), of amino acids collected from parotid saliva making cationic amino acids the most variable in terms of concentrations in saliva.(18).
   There is another possible pathway which explains cationic amino acids relationship to dental caries and which involves the NIS. The two primary transport systems for cationic amino acids are the y+,"classic", system and the y+L system.(19,20). In the presence of an inwardly directed negative diffusion potential as can be created by the inwardly directed gradient of anions such as thiocyanate, SCN^-, the influx of lysine increases. A number of studies have demonstrated an increased influx of arginine and lysine in the presence of membrane hyperpolarization that can be induced by the transport of anions.(21,22,23). The increased influx is thought to be due to conformational changes in the y+ carrier and increased negative surface membrane potential in the y+L carrier.(24,25).
   Having identified a mechanism to explain an increase in the concentration of cationic amino acids in saliva it is important to also identify those anions other than SCN^- which have the ability to hyperpolarize the cell membrane during transport. S. Eskandari et al. have shown that I^-, SCN^-, $SeCN^-$, ClO_3^-, and NO_3^- are all preferentially transported by the NIS. These anions are concentrated by the salivary gland in a S/P ratio much greater than unity. They also all have the

ability to hyperpolarize the cell membrane, creating a negative membrane potential and that this can result in an increased transport of the cationic amino acids, arginine and lysine.(26). Again in the human placental membrane Boyd and Vatish have shown that anions create an inside negative potential with an increased influx of lysine and it is proportional to their relative permeability's with $SCN^- > I^- > NO_3^-$.(27). From the results of the study by B.C. VanWuyckhuyse et al. and L. Vranic et al. we may infer that one or more of these anions has cariostatic capabilities.

  The NIS has been demonstrated in ductal cells by its immunoreactivity to anti-NIS antibodies.(28). In immunohistochemical studies of the human NIS, hNIS proteins were detected in the ductal cells.(29,30). The duct system of the salivary glands progresses from the acinar lumen to the intercalated ducts, the intralobular striated ducts, and last we have the interlobular ducts which empty into the oral cavity. The striated ducts are the most vascularized of all the ducts. The NIS has been identified in the striated duct and is in close proximity to the cationic amino acid transporters. The parotid gland contains the most extensive striated duct system of all the major salivary glands.(31,32).

  This along with the previously presented information supports the theory that 1) the NIS plays a key role in determining an individual's susceptibility to caries and 2) that one of the anions, I^- ,ClO_3^-,NO_3^-,SCN^-,$SeCN^-$, transported by the NIS or a combination of them are factors in this process. It appears that the levels of free arginine and lysine in saliva may serve as markers for the transport of these cariostatic anions by the NIS and for an individual's susceptibility to caries.

Sexual Dimorphism, the NIS, and Dental Caries

  Another indication of the role of the NIS in dental caries is the well documented sexual dimorphism of dental caries. Females have a much greater caries experience than males in permanent teeth. In studies conducted prior to the fluoridation of community water supplies we find 24 studies involving the sexual dimorphism of dental caries. 21 studies or 87.5% support the concept of increased caries in females, two studies, 8.3%. found no difference between the two sexes, and only one study indicated a higher rate for males, 4.2%.(33). In an analysis of caries experience among 12 to 17 year olds covering the years 1946 to 1959 encompassing 9 studies it was determined that females had a decay rate 16.8% higher than for males. It was also determined in 9 studies of 12 to 17 year olds in the years from 1983 to 1993 that the rate of caries was 13.7% higher in females.(34). In addition the rate of caries increase with age is greater with females than with males.(35,36,37). Anthropologists will generally attribute these differences to behavioral factors and variation of roles such as food preparation as having an impact on caries rates. Dental anthropologist J. Lukacs has suggested that biological factors including sex hormones, estrogens, particularly estradiol, play a key role in these differences although specific mechanisms are not described.(33,35,38).

  Estradiol also known as oestradiol, E2 or 17 *b*-estradiol is the major sex hormone found in females. It can be found in males as a metabolic product of testosterone. It along with estrone, E1, and estriol, E3, comprise the three major estrogens produced by the follicular cells in the ovaries, corpus luteum, and the placenta. Serum levels of estradiol reflect the activities of the ovaries.

  Throughout puberty there are significant increases in the serum levels of estradiol in girls. These increases continue on through adulthood. Table 2 demonstrates this trend with estradiol levels divided into Tanner stages to quantify sexual development. We see a significant increase in E2 production, especially from stage V to adulthood.(39,40). In examining the levels of serum E2 in boys the rise of serum levels is steady and much less dramatic than that seen in girls, Table 3. The adult levels in males, avg. 68 pmol/L, (n=18), is very similar to those seen in level V.(41).

Table 2 (Modified from table 1, ref. 39)

Tanner Stage	I	II	III	IV	V	Adult
Age(yr)	9.1	11.4	12.2	14.5	16.8	26.6
Estradiol(pmol/L)	22	40.5	128.5	162	182	289

Girls and women

Table 3 (Modified from table 1, ref. 41)

Tanner Stage	I	II	III	IV	V	Adult
Age(yr)	9.7	12.6	13.6	15.7	17.3	25-60
Estradiol(pmol/L)	<18	21	36	59	71	68

Boys and men

 This reflects the pattern described earlier in the increasing divergence of caries rates in females over males. Because E2 serum levels reflect the activity of the ovaries we see a dramatic increase in activity in the menstrual cycle. It acts as a growth hormone for the female reproductive organs.(39).

Table 4 (Modified from table 2, ref. 39)

Menstrual Stage	Early Follicular	Late Follicular	Periovulatory	Midluteal	End Luteal
Estradiol (pmol/L)	164 (74-611)	425 (113-1509)	330 (171-955)	436 (75-1025)	250 (<18-864)

   Pregnancy brings about marked increases in estradiol levels, there is a steady increase in serum levels throughout pregnancy. As a reference, by the eighth week of pregnancy estradiol levels are two to three times the highest concentrations seen in the normal menstrual cycle.(42). Similar results were found in a longitudinal study of selected individuals with E2 levels at the 6th week twice those found at the mid-luteal phase of the menstrual cycle. There is a rapid increase at the 8th week due to the initiation of placental production of the hormone.(43).
   A proverb in Germany, Russia, Denmark, and Japan states, "for each child a tooth". A study in Denmark of 34 pairs of identical females twins compared tooth loss with children borne. Regardless of social status the number of missing teeth increased with number of children.(44). A study conducted of women in Gothenburg, Sweden compared number of children with remaining teeth. There was an increased risk of being edentulous with increased number of children.(45). These studies do not differentiate tooth loss from periodontal disease or dental caries. A study conducted comparing pregnant with non-pregnant women in Chaing Mai, Thailand found that there was a significant difference, (p<0.001), in dental caries.(46).
   An interest in estradiols effect on the NIS has stemmed from the sexual dimorphism exhibited by thyroidal goiters. In a 1977 survey of Whickham, England overt hypothyroidism was present in 14/1000 females compared with 1/1000 in males. Goiters were four times more common in females than males, more prevalent in younger than older women.(47). In a 20 year follow up of the original study new cases of spontaneous hypothyroidism were 3.5/1000/year in females but only 0.6/1000/year for men.(48). Another study indicates 16% of young women had sporadic diffuse goiters and states the peak occurrence of this condition occurs during pregnancy, possibly reflecting an iodide deficiency.(49). As early as 1917 D. Marine stated, " the periods when thyroid enlargements most frequently occur are at puberty, during menstruation, and during pregnancy".(50,51).
The clinical expression of a state of hypothyroidism due to decreases in iodide transport is a goiter. A goiter is defined in clinical terms as an enlargement of the thyroid gland by a factor of from 4 to 5. The main cause of goiters is a lack of iodide in the diet which may be aggravated by goitrogens, medications or substances that inhibit iodide uptake or metabolism. Even in areas

where there is the prophylactic use of iodine there is a persistence of goiters suggesting the role of goitrogens. Goiters are more prevalent in females than males even when there is adequate iodine in the diet.(52).

Research on estradiol's direct effect as a goitrogen on thyroid follicular cells has been conducted by T.W. Furlanetto et al.(1999), with the use of FRTL-5 cells, Fisher rat thyroid cell line. It was demonstrated that E2 has the ability to inhibit the TSH stimulated expression of the NIS gene. This may explain the effect of E2 on the thyroid but as mentioned previously the salivary glands are not under the influence of TSH. (53). In a second study by T. Furlanetto et al.(2000) it was determined that E2 decreased iodide uptake by the FRTL-5 cells in the presence and absence of TSH. The direct effect of E2 on the thyroid cells was expected as estrogen receptors have been identified on thyroid follicular cells, Figure 7 .(54).

This data would support the increased presence of goiters in females. Also, if as already presented we accept the fact that the NIS is responsible for the transport of cariostatic substrates into the saliva this data supporting estradiols role as an NIS inhibitor explains the sexual dimorphism of dental caries rates in females. There are papers that take the point of view that systemic diseases and conditions such as pregnancy have no impact on dental caries.(55).
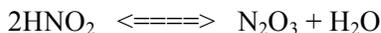
Nitrosation

There is a chemical process which can take place in saliva that involves at least three of the anions transported by the NIS. Nitrosation is a process that involves the formation of nitrosocompounds symbolized as R-NO. Nitrosation has been studied intensely as it is thought by some that the formation of N-nitrosocompounds may result in cancer. Cortas and Wakid have demonstrated that there is a direct correlation between serum levels of nitrate, NO_3^-, and salivary nitrate and nitrite, NO_2^-, levels. Serum nitrate was shown to be concentrated by a factor of 9 by the NIS in saliva. Nitrate is detected in salivary gland secretions but nitrite is not. Nitrite in the oral cavity is the result of the reduction of nitrate by oral bacteria, facultative anaerobes, that reside on the posterior dorsal surface of the tongue and bacteria in saliva.(56,57). In addition reduction of nitrate to nitrite has been shown to occur in plaque.(58). Nitrate is a relatively stable anion with a low degree of reactivity but nitrite has the ability to react with weak acids forming nitrous acid which can play a role in a number of reactions including nitrosation and the formation of nitric oxide.(59,60,61). Nitrite in the presence of a mild acid such as lactic acid forms nitrous acid, HNO_2.

$$NO_2^- + {}^+H_3O <====> HNO_2$$
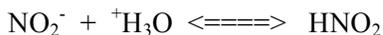
Nitrous acid is a weak acid with a pKa of 3.14 but is responsible for numerous nitrosation reactions.

Bacteria that have been implicated in causing dental caries include S. salivarius, S. mitis, S. sanguis, and S. mutans which colonize pits and fissures first. The more virulent cariogenic bacteria, S. mutans, Lactobacillus including L. casei which can decrease the pH to as low as 3.2 and possibly actinomycetes are later colonizers and thought to be the bacteria mainly responsible for caries.(62,63,64). Jensen and Wefel have demonstrated in vivo that the interproximal plaque pH can reach a level as low as 3.69 +/- 0.12 after a meal of fermentable carbohydrates.(65).

HNO_2 exists in aqueous solution in equilibrium with N_2O_3, dinitrogen trioxide, that can act as a nitrosating agent:

$$2HNO_2 <====> N_2O_3 + H_2O$$

The active agent in nitrosation reactions is the nitrosonium ion, NO^+. NO^+ is an active agent mainly through the activity of carrier molecules such as dinitrogen trioxide, N_2O_3 which can be

depicted as $NO_2^-NO^+$, dinitrogen tetraoxide, N_2O_4 depicted as $NO_3^-NO^+$, nitrosothiocyanate, ONSCN depicted as $SCN^- NO^+$, or S-nitrosoglutathione, GSNO depicted as GS^-NO^+.

N_2O_3 may react with a number of substrates, the most researched being secondary amines and amides. This process of nitrosation has been shown to be present in whole saliva. Nitrosamines were formed when the secondary amine morpholine was added to saliva even at neutral pH and the process increased as the pH decreased. Nitrite was shown to be present in the saliva taken from subjects and salivary catalysts were implicated in this process.(66). G. Rao points out that even though the formation of nitrosamines is unlikely at neutral pH there are local conditions in plaque where the pH may be lower and anions such as thiocyanate may contribute to the process of nitrosation.(67). Other substrates include the amino acids cysteine and proline. Nitrosation may proceed by an electrophilic attack by the nitrosating agent on N,O,C, or S compounds. Those compounds that contain a sulfhydryl group, -SH, are known as thiols and the products of their nitrosation are termed S-nitrosothiols. Of these nucleophilic centers protein sulfhydryl groups have been shown to be the most reactive and will be preferentially nitrosated.(68). S-nitrosation has been shown to take precedence over N-nitrosation when both amines, amides and sulfhydryl groups are exposed to nitrosating agents.(69,70). The sulfhydryl group of cysteine plays a key role in the catalytic process of many enzymes due to its electron donating capabilities. The bacteriostatic effect of acidified nitrite involves the nitrosation of sulfhydryl, -SH residues in enzymes or carrier proteins. Once nitrosated these enzymes reversibly or irreversibly lose their catalytic capabilities.(71).

The reaction of N_2O_3 with substrates(S) is given as:

$$2\ HNO_2 \quad \overset{K}{<====>} \quad N_2O_3 + H_2O$$

$$N_2O_3 + S \overset{k}{-------->} S^+\text{-}NO + NO_2^-$$
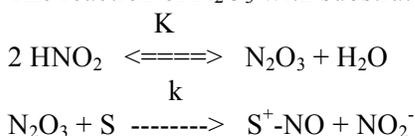
Because the substrates in the caries models which will be examined later are very reactive their reaction with N_2O_3 will be faster than its hydrolysis to nitrous acid, the second order rate limiting step becomes the formation of N_2O_3 or:

$$Rate = k'(HNO_2)^2$$

There are other nitrosating agents besides N_2O_3. Many of these reactants may be formed from nitrous acid and act as catalysts in this process. Nitrosyl halides can be formed from chloride, bromide, iodide, but fluoride has not been found to be involved.

$$HNO_2 + X^- + H^+ \quad <====> \quad XNO + H_2O$$
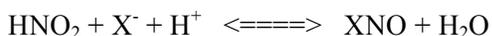
As with the nitrosation reaction of N_2O_3 their formation becomes the rate limiting step in their action as catalysts. Nitrosyl iodide is a very effective nitrosating agent compared with either chloride or bromide based on the nucleophilicity of the agents. The most effective catalyst is thiocyanate, SCN^-. The ratio of reactivity for the representative substrate morpholine is 1:30:15,000 for chloride, bromide, and thiocyanate respectively.(59).

$$HNO_2 + SCN^- + H^+ \quad <====> \quad ONSCN + H_2O$$

$$ONSCN + S \quad ----> \quad S\text{-}NO + SCN\text{-}$$
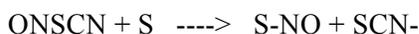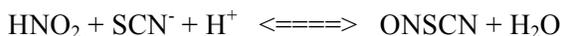
ONSCN has been shown to be formed rapidly in acidified saliva, pH 5.2, from $NaNO_2$,(0.2 mM), and NaSCN,(1 mM), all parameters well within physiologic limits.(72).

Studies have compared the nitrosation of the amino acids analine, glycine, and valine by various nitrosating agents. These agents were N_2O_3, ONCl, ONBr, ONSCN, and thiourea,$SC(NH_2)^2$. Nitrosothiocyanate, ONSCN, was an effective nitrosating agent with the half-life for the reaction dropping from 4000 sec. for N_2O_3 to approximately 5 sec. with the addition of SCN^-.(73).

This paper will demonstrate a strong connection between the process of nitrosation and dental caries. It appears that nitrous acid, a product of nitrate, plays a key role in the process. Also nitrosation proceeds in the second order in nitrous acid and nitrite concentrations. Of the catalytic anions thiocyanate has been identified as the most effective followed by iodide, bromide, and chloride.

Because the catalyzed pathway is first order in nitrite and thiocyanate concentrations nitrosation will be proportional to their concentrations. In the uncatalyzed pathway N_2O_3 is related to the square of the nitrite concentration therefore ONSCN may have a greater impact at lower nitrite concentrations, N_2O_3 at higher nitrite concentrations.(74).

Nitrate, Thiocyanate, and Iodide Concentrations in Saliva

Both nitrate and thiocyanate are present in millimolar concentrations in saliva. Approximately 85% of nitrate acquired by the body is from vegetables, specifically those categorized as green, leafy vegetables.(75). Nitrate from beverages contribute approximately 13% of dietary nitrate most coming from the nitrate levels in water. The remainder may result from the addition of nitrate to foods, specifically processed meats, as an antibacterial agent against Clostridium botulinum.(76). There can be considerable variations in these figures, especially in regions where there are high nitrate levels in water supplies resulting in its contribution increasing to as much as 50% of the total.(77). The dietary nitrate is absorbed in the proximal small intestines and at least 25% is transported into saliva by the NIS. Nitrate is absorbed very efficiently resulting in a 100% bioavailability.(78). Approximately 20% of the nitrate is reduced to nitrite by facultative anaerobes involving nitrate reductase in the anoxic environment on the posterior dorsal surface of the tongue, dental plaque, and in saliva. There can be as much as a three fold variation in individuals conversion abilities.(79). A paper by J. Doel et al. that first focused my attention on nitrate and caries analyzes individuals nitrate reductase abilities as it relates to DMFT. In 209 children it was determined that those with higher nitrate/nitrite reduction capacity had a significantly lower caries experience.(80). This process results in approximately 5 to 7% of ingested nitrate being converted to nitrite but can be as high as 20% for some individuals.(81,82,83,84). Approximately 80% of salivary nitrite is derived from the process of nitrate reduction in the oral cavity, the other 20% being ingested in foods and beverages.(85). Nitrate once ingested will reach a peak level in approximately 1 hour if in a liquid form, such as vegetable juice, and from 2-3 hours if in a solid form as in vegetables. Nitrate not converted in the oral cavity will recirculate resulting in a second or even third maxima of salivary nitrite before finally being excreted.(81). The half-life of nitrate in the serum is estimated at 12 hours by S.R.Tannenbaum et al. but as short as 5 to 8 hours by M.Tonacchera et al.(79,86).

Thiocyanate originates from either smoking or particular vegetables. Cigarette smoke is detoxified in a process in which cyanide reacts with thiosulfate to form thiocyanate, the reaction is catalyzed by thio-sulfate-cyanide sulfurtransferase,(Rhodanese). Smokers will have a 3 times greater concentration of thiocyanate in their saliva. A few plants such as cassava, lima beans, sweet potatoes, maize, and sorghum contain cyanogenetic glucosides which is then detoxified to thiocyanate. The Brassicaceae; cabbage, cauliflower, turnips, broccoli, brussel sprouts, and rutabaga, follow a different metabolic pathway and are hydrolyzed to isothiocyanate, nitriles, and thiocyanate.(87,88). Thiocyanate in saliva has a more consistent concentration than nitrite due to its longer serum and salivary half- life which can be 10 to 14 days.(89,90,91).

Iodide may have a minimal impact on the process of nitrosation in the oral cavity. A comparison of thiocyanate and iodide shows a thousand fold greater concentration of thiocyanate

in saliva versus iodide on an eqimolar basis, 1.2 +/- 0.7 mM and 1.8 +/- 1.1 X 10^{-3} mM respectively in non-smokers.(92). Another study found the salivary molar iodine ion concentration to be .7 X 10^{-3} mM.(93).

Cationic amino acids in saliva will reflect increases in the passage of not only larger amounts of nitrates but also thiocyanate, both of which play an active role in nitrosation. Nitrate has been shown as being a necessary component in nitrosation, with thiocyanate an important factor in the role as catalyst. Although the elevated level of thiocyanate in smokers might appear to be a benefit to the process of nitrosation we will find that due to its competitive antagonism with nitrate in their transport by the NIS smokers will have lower levels of nitrate and nitrite in saliva. This will inhibit the nitrosation process in these individuals and be reflected in their higher caries rates, a process that will be discussed later.

Hormonal control of the NIS may be reflected in lower concentrations of nitrate and thiocyanate in the saliva of women as well as lower nitrite concentrations. A study by S.S. Mirvish et al. found women to have a lower nitrate concentration in their saliva, 23 mg/l, versus men, 35 mg/l. In addition women also had a lower salivary nitrite concentration, 7.5 mg/l versus men, 9.9 mg/l.(94).

In a study with 100 participants, 50 men and 50 women, salivary nitrite levels were less for females than for males, 28 +/- 4 mg/L versus 33 +/- 5 mg/L although not of statistical significance.(95).

Tenovuo and Makinen found a higher concentration of thiocyanate in the saliva of male nonsmokers versus female nonsmokers, 91.8 +/- 43.7 mg/L and 61.7 +/- 31.6 mg/L respectively. The same pattern held for smokers who have a higher thiocyanate concentration, 210 +/- 75.0 mg/L and 124 +/- 46.3 mg/L respectively.(92).

Chronic Renal Failure and Nitrates

In a review of the literature there is a connection between two of the NIS anions and a disease process. The involvement of these anions is reflected in a decreased rate of caries in Chronic Renal Failure,CRF, patients.

M. Chow et al. (1979) reported on a case study of an 8 year old with CRF. It was recognized that the child had an extremely low rate of dental caries in spite of the fact that he was on a high carbohydrate diet to supplement a required low protein diet. The low rate of caries was attributed to an elevated level of urea seen in the saliva of patients with CRF.(96). In 1985 there are three studies on children with CRF, all displayed a significant reduction in caries rates when compared with controls with the results attributed to elevated urea levels.(97-99). Additional studies on CRF patients have all demonstrated a reduction in caries rates.(100-104).

These studies on CRF patients are often referenced in other studies on the cariostatic capabilities of urea. Of the nitrogenous compounds in saliva arginine and urea are considered the main sources of ammonia, which is in turn credited with creating a more alkaline environment. Free arginine is catabolized by the arginine deiminase system, ADS, resulting in ornithine, carbon dioxide, and ammonia. The study by VanWuyckhuyse et al. is often referenced in association and justification of the concept that free arginine levels are factors in certain individuals caries resistance.(105). Urea which is present in salivary levels that reflect serum levels is hydrolyzed by bacterial ureases to carbon dioxide and ammonia.

In CRF patients there are elevated levels of two of the cariostatic anions identified earlier. In a study on hypotensive episodes during hemodialysis M. Nishimura et al. identified elevated levels of nitrate in the serum of CRF patients. We see in CRF a disease process in which, due to a decrease in renal clearance, there is an increase in serum nitrate levels. This circulating NO_3^- can only be removed from the serum of CRF patients by dialysis. The study shows pre-dialysis levels of NO_3^- as avg. 307 umol/L versus avg. 133 umol/L following treatment.(106). This elevated nitrate level in the serum will be reflected in an elevated salivary nitrite level.

T. Blicharz et al. researched the possibility of using colorimetric test strips to monitor a patient's progress while on dialysis. It was discovered that the two analytes that decreased significantly during dialysis in terms of salivary concentrations were uric acid and nitrite. Test strip measurements showed a decrease of 39% for uric acid and 86% for nitrite during the course of treatment.(107).

In a study by A. Cailleux et al. serum levels of thiocyanate, SCN^-, were determined in controls and CRF patients. The controls were divided into smokers and non-smokers as serum thiocyanate levels are higher in smokers. The SCN^- levels of smokers were 206+/-74 umol/L, very close to 3 times the level of non-smokers, 74+/-19 umol/L. Non-smoking CRF patients had levels of 91+/-24 umol/L prior to dialysis and 62+/-21 umol/L following treatment. The elevated levels of SCN^- in CRF is again due to impaired renal clearance as with nitrates.(108).

K. Koyama et al. have also shown elevated serum SCN^- levels in preterminal chronic renal failure patients, (PCRF), and also those on dialysis. PCRF patients and pre-dialysis patients had SCN^- levels approximately double those of the controls.(109).

Sjogren"s Syndrome, Nitrates, and Xerostomia

There is a disease process which may reflect an increase in caries in conjunction with a decrease in salivary nitrite and xerostomia. Numerous studies have found a higher caries rate in Sjogren's Syndrome, (S.S.), patients when compared with controls.(110-113). S.S. is an autoimmune disease affecting the salivary and lacrimal glands, is present in from 1% to 3% of the population and affects nine times more females than males.(114). In S.S. there is a lymphocytic infiltrate of the salivary and lacrimal ducts with a pronounced alteration in the functioning of the striated salivary ducts involving hyperplasia of the basal cells and aberrant differentiation into stratified and reticulated epithelium.(115). Another important symptom is a dry mouth or xerostomia which only becomes evident when salivary flow drops below 50%. (116). Also the saliva in S.S. patients has a more viscous and foamy nature as opposed to the serous consistency of the healthy controls.

D. Xia et al. studied nitrate and nitrite concentrations in parotid and whole saliva in S.S. patients and controls.(117).

Table 5 (Modified from table 2, ref. 117)

		Sjogren's Syndrome	Control
Concentration (mg/L) nitrate	Parotid saliva	53 +/- 34	172 +/- 82
	Whole saliva	24 +/- 15	97 +/- 47
Concentration (mg/L) nitrite	Parotid saliva	Undetectable	Undetectable
	Whole saliva	6.8 +/- 3.3	10.4 +/- 5.8

The nitrate concentration in whole saliva is significantly less than the controls leading to a decreased concentration of nitrite in whole saliva. T. Granli et al. have shown that nitrate concentrations in saliva are inversely proportional to salivary flow.(118). In S.S. we find a significant decrease in the concentration of nitrate in whole saliva in spite of a decreased salivary flow. This is in conflict with the results of Granli indicating an impairment of the transport of nitrate by the cells of the striated duct. This is also indicated by a study where some S.S. patients show a lower saliva/plasma iodide ratio at given flow rates compared with normal subjects. These same individuals exhibit the most severe radiological changes on sialographys.(119). The increase in caries rates in S.S. patients may be due to the combination of a decrease in nitrate and nitrite concentrations in saliva combined with a decreased salivary flow rate. The exposure to the cariostatic agent can be given as mg/min. We reach this number by multiplying concentration,

(mg/l), by flow rate,(ml/min.). The exposure of nitrite for the controls is 31 +/- 17 mg/min. and for the S.S. patients, 7 +/- 4 mg/min. in whole saliva.

   B. Zeldow discovered that the antimicrobial activity of parotid saliva was greater than that of the submandibular gland in 24 of 25 patients.(120). According to Granli et al. the nitrate concentration of unstimulated parotid saliva is 2.8 times greater than the concentration of nitrate plus nitrite found in unstimulated whole saliva. Following the bilateral ablation of the parotid glands in miniature pigs D. Xia et al. determined that the background concentration of nitrate was approximately 3 times greater in the controls than the test subjects. Also the concentration of nitrate following a nitrate load was significantly less in the test subjects indicating the parotid glands importance in the transport of nitrate from serum to saliva.(121). SCN^- has also been determined to be more concentrated in parotid saliva versus the submandibular gland at flow rates between 0.1 and 1.0 ml/min.(122). R. Harden et al. have shown that the iodide clearance is greater in the parotid gland, 5.7+/- 0.50 ml/min., than submandibular gland, 3.4+/- 0.50 ml/min.(123). Harden et al. found more iodide was excreted by the parotid than submandibular gland, 0.62 +/- 0.115 ug/hr versus 0.27 +/- 0.051 ug/hr respectively.(8). A more foamy and viscous saliva has been associated observationally if not scientifically with dental caries. Because the parotid gland produces a more serous saliva as opposed to the other glands which have a more mucous component any decrease in the capability due to damage of the parotid gland will result in this more viscous and foamy saliva, most likely with a decreased nitrate component.

Radiation Therapy, Nitrates, and Xerostomia

   Radiation therapy creates another situation involving an increase in caries rates similar to that seen in S.S. Caries rates increase with the initiation of radiation therapy, R.T.(124-126). Radiation caries only occurs when the salivary glands are within the path of the radiation and do not occur if it is only the teeth that are exposed.
   As with S.S. patients there is a significant decrease in salivary flow. S. Dreizen et al. documents a decrease from an unstimulated flow rate of 1.1 mL/min. to 0.47 mL/min. within the first week of therapy. The flow rate declined gradually after this to .05 mL/min. over the next 3 years. The loss of the ability to produce saliva is progressive and non-reversible. This loss parallels the nonregenerative destruction of the acinar cells of the gland.(127). Exposure of minipig salivary glands, chosen because of their physiological and histologic similarities with human salivary glands, to 70 Gy of ionizing radiation promotes a specific form of destruction of the glands. The parotid gland following irradiation was approximately half the size of a normal gland. There is acinar atrophy with striated duct dilation and an infiltration of lymphocytes and plasma cells denoting chronic inflammation.(128).
In a study involving 207 head and neck cancer patients xerostomia was considered the most common oral sequele with up to 80% of patients complaining of a dry mouth.(129).
   C. Chen et al. monitored 15 patients undergoing radiation therapy for nasopharyngeal carcinoma. Again as with S.S. the salivary flow rates decreased significantly from 4 ml/min. at baseline to 1.7 +/- 0.7 at 10 treatments,RT10, 1.3 +/- 1.0 at 20 treatments,RT20, and 1.1 +/- 1.2 at the endpoint. There was not only a decrease in the nitrate concentrations in saliva but also a decrease in salivary nitrite concentration.

Table 6 (Modified from table 2, ref.130)

	Baseline	Endpoint
Salivary nitrate	1.11 +/- 1.32	0.31 +/- 0.36
Salivary nitrite	0.17 +/- 0.14	0.13 +/- 0.17

Again as with S.S. the decrease in salivary flow should lead to an increase in nitrate concentrations but instead there was an overall decrease indicating damage to the secretion process in the salivary glands, all the major glands were involved.(130).

The Effect of Smoking on the NIS and Dental Caries

In 1951 Ludwick and Massler conducted a study on naval enlistees to determine the relationship of caries to smoking. They were expecting to achieve the same results as those of H.J. Schmidt,(1951), who reported that increases in tobacco smoking led to a decrease in caries rates. Instead they found that in all age groups there was a statistically significant increase in caries rates with cigarette consumption.(131). This positive correlation between increased cigarette consumption and caries rates has been demonstrated in a number of studies.(132-135).

Having identified smokers as being at risk for developing dental caries we will discuss these results in terms of the cariostatic anions identified earlier. Smoking leads to increased levels of thiocyanate in serum and in saliva. Tenovuo and Makmen recorded thiocyanate in the saliva of smokers and non-smokers. Smokers had whole saliva concentrations of 2.6+/- 1.3 mM/L whereas non-smokers had 1.2+/- 0.7 mM/L. The concentrations in whole saliva varied by cigarette consumption per day.(92).

Table 7

Cigarettes/day	SCN^- (mg/L)
0-5	95
6-10	163
>10	194

N. Haley et al. document plasma SCN^- concentrations of smokers at 157+/- 34 uM/L and non-smokers at 57+/- 22 uM/L. Whole saliva concentrations were 3.339 +/- 1.117 mM/L and 1.293 +/- 652 mM/L respectively indicating that SCN^- is concentrated by a factor of 21 and 22 through the activity of the NIS in the salivary gland in this study.(89).

Thiocyanate plays a dual role in the dental caries process. At higher concentrations it has the ability to competitively inhibit the transport by the NIS of other anions such as nitrate and iodide from serum to saliva. At lower concentrations of SCN^- it may act as a catalyst in the process of nitrosation.

Smoking has been shown to inhibit the transport of iodide by the NIS in various in vivo studies. L. Hegedus et al. conducted a study to determine smoking's impact on the frequency of goiters. The incidence of goiters was higher in smokers, 30% versus 3% among non-smoking participants.(136). Thiocyanates ability to inhibit the transport of iodide is demonstrated by decreasing concentrations of iodide in saliva as the SCN^- concentration increases in smokers versus non-smokers.(92).

In a similar manner thiocyanate has the ability to inhibit nitrate transport by the NIS. In a study by Ladd and Archer it was found that as salivary levels of SCN^- increased in smokers the levels of salivary nitrite decreased. The participants were divided into three groups, non-smokers, light smokers,(<30 cigarettes/day), and heavy smokers,(>30 cigarettes/day). Heavy smokers exhibited less than half the salivary nitrite concentration of nonsmokers.(137,138).

C. Cingli et al. in a study with 100 participants found that the nitrite levels in whole saliva was greater in nonsmokers, 35 +/- 5 mg/L, compared with smokers, 21 +/- 4 mg/L.(p<0.001).(95).

D. Forman et al. compared the nitrate and nitrite levels in populations with a low and high risk of developing gastric cancer in Great Britian. They found lower levels of nitrate in the saliva of smokers, 135.7 nmol/ml versus 172.0 nmol/ml in non-smokers in the low risk population. In

the high risk population smokers had a salivary level of nitrate that was 85.8 nmol/L versus 116.6 nmol/L in the non-smokers. Salivary nitrite levels in the low risk category followed this same pattern with 87.9 nmol/ml in smokers and 104.6 nmol/ml in non-smokers. In the high risk population smokers had a level of 53.7 nmol/ml nitrite while non-smokers had 73.6 nmol/ml. These results were not expected if we are to assume that nitrates contribute to the formation of carcinogens in the form of N-nitrosocompounds as smokers have an elevated risk of gastric cancer.(139).

The Effect of Secondhand Smoke on the NIS and Dental Caries

   Not only smoking but secondhand smoke appears to have an impact on caries rates. Various studies have demonstrated a dose dependent relationship between increased exposure to environmental tobacco smoke,ETS, and increased caries rates.(140-142).
   It is well documented that there is an increased level of SCN⁻ in the saliva of smokers versus non-smokers. L. Krebs undertook the task of identifying these levels in individuals exposed to secondhand smoke.

Table 8 (Modified from ref. 143)

Smoke Exposure	SCN- in saliva (mM)
Nonsmoker lives with nonsmoker	1.16
Nonsmoker lives with nonsmoker	1.24
Nonsmoker lives with nonsmoker	1.27
Nonsmoker works with smokers	1.71
Nonsmoker lives with smoker	1.83
Nonsmoker lives with smoker	2.30
Nonsmoker lives with two smokers	4.34
Smoker (1/2 pack a day)	2.12
Smoker (1/2 pack a day)	2.14
Smoker (1 1/4 pack a day)	3.78

   From Table 8 we see that there are progressively greater levels of SCN⁻ with exposure to secondhand smoke from non-smoker, (lives with non-smoker), to non-smokers,(works with smokers), to non-smokers,(lives with two smokers). The level of SCN⁻ in the saliva of the last individual is greater than that of a 1 ¼ pack a day smoker.(143). The increased levels of SCN⁻ in those exposed to secondhand smoke would explain the increase in caries rates when placed in the context of its inhibitory capability on the transport of nitrate by the NIS.

Lithium, the NIS, and Dental Caries

   Another documented goitrogen besides thiocyanate and estradiol is lithium. It may be that lithium, Li^+, substitutes for the Na^+ in the Na/I symporter although it is not nearly as effective as the sodium ion in this role.(26). Lithium salts have been used since the 1960's for the treatment of bipolar or manic depressive disorders. The usual regiment is a daily dose of from 0.6 to 2.4 gm of lithium carbonate. It is concentrated in saliva by a factor of from 3 to 5 over serum levels. Approximately 20% to 30% of patients develop hypothyroidism. The caries rates in these individuals are elevated although poor home care and xerostomia caused by medications become confounding factors.(144,145).

Thiocyanate

The first indication of thiocyanates role in caries reduction was presented by J.W. Beach in 1908 based on salivary analysis for sulfocyanate, an early term for thiocyanate. A Point of Care test for caries susceptibility was proposed to determine thiocyanate levels in saliva.(146).

Research conducted in the 1960's identified what was termed a bactericidin system in saliva which was active against L. acidophilus ATCC 4357. Of the anions iodide, chloride, bromide, sulfate, thiosulfate, thiocyanate, nitrate, and phosphate, only iodide and thiocyanate reinstated bactericidal action to dialyzed saliva. Only concentrations of iodide,(> 0.175 mg/100ml), greater than that found in normal saliva,(0.0035-0.024 mg/100ml), were effective and salivary levels of iodide did not potentiate thiocyanates capability. Thiocyanate in concentrations found in saliva,(1-27 mg/100ml), had a dose dependent ability to restore the antimicrobial system in dialyzed saliva. Concentrations of thiocyanate that were eight times that found in saliva were toxic to bacteria without other salivary components. Patients were given 500 mg potassium perchlorate, a potent NIS antagonistic inhibitor, and the saliva that was collected one hour later showed a decrease in antimicrobial activity demonstrating the importance of the NIS in the system. In addition patients given 500 mg potassium thiocyanate exhibited an increase in thiocyanate levels in their saliva one hour later compared with levels one hour prior to administration, with a corresponding elevation of antimicrobial activity. The inability of nitrate to potentiate the system is understandable as Lactobacillus lack the ability to reduce nitrate to nitrite.(147). Similar results were found in a study involving L. casei with thiocyanate at salivary concentrations reinstating the antibacterial effect of dialyzed saliva but iodide was ineffective at normal salivary concentrations.(148).

Not only thiocyanate but also isothiocyanate may act as a catalyst in the nitrosative process. Isothiocyanate, ITC, has the chemical structure, $S=C=N-R$. Miswak is included in a list of 18 natural substances that have anticariogenic properties although its mode of activity is noted as unknown.(149). It is an ancient form of a toothbrush known as a cleaning stick dating back to the Babylonians. Roots of the Salvadora Persica, a small tree or shrub are soaked and chewed, shredding the tip to form a rudimentary toothbrush. Miswak is currently used from Africa through the Middle East and Asia.(150,151). The essential oil from the root is 90% benzyl isothiocyanate,(BITC), according to one report.(152). Another reported levels of 69.5%.(153).

Wasabi is known as Japanese horseradish. In literature for public consumption that highlights foods that protect the teeth wasabi is generally included. Isothiocyanates are the main volatile component of wasabi and contribute to its distinct pungent odor and taste.(154).

In a study examining its impact on dental caries Wistar rats were fed a diet which included isothiocyanate,(50 ppm). The effect was antibacterial against mutans streptococci and the inhibitory action increased with longer side chains, R , on the thiocyanate ion.(155). ITCs may also be released from raw cruciferous vegetables when chewed, the action catalyzed by the enzyme myrosinase contained in the vegetables. The enzyme converts glucosinolates in the vegetables through hydrolysis to ITCs. These vegetables include broccoli, Brussel sprouts, cabbage, cauliflower, collard greens, kale, and turnips.(156).
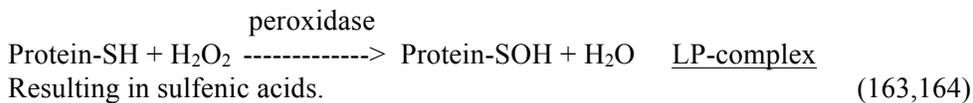
In a study by S. Morris et al. it was shown that the addition of the R moiety to nitrosothiols structure has a positive impact on the effectiveness of the nitrosating agent. The addition of an electron withdrawing group contributes to the nucleophilicity of the sulfur atom. Effectiveness of S-nitrosothiols, $RSN=O$, on the nitrosation of sulfhydryl groups of Bacillus cereus during germination, causing an inhibition of spore outgrowth, increased as the electron-withdrawing nature of the R group increased.(157).

Oxidative/Nitrosative Stress

Klebanoff and Luebke identified salivary peroxidase and thiocyanate as components in what was termed the antibacillus system.(158). The lactoperoxidase-thiocyanate-H_2O_2, LP-complex, was found to be operative in milk and saliva.(159,160). The major oxidizing agents at the cellular level include hydrogen peroxide,(H_2O_2), the superoxide radical,($O2^-$.), and the hydroxide radical,(OH^-). Research has included the food sciences as starter cultures of lactic acid bacteria are instrumental in dairy products and their inhibition by the LP-complex was an issue. The LP-complex is analogous to the process of nitrosation, the difference being one involves oxidative stress on sulfhydryl,-SH, residues in enzymatic proteins whereas the other involves nitrosative stress. Oxidation of sulfhydryl groups in bacteria has been directly linked with a loss of viability.(161). The term nitrosative stress was coined by J.S. Stamler (1999), to denote products of oxidative stress formed by reactive nitrogen species,(RNS), i.e. N_2O_3, N_2O_4, NO^-, and NO_2^- .(162). Both nitrosative and oxidative stress use reversible 2 electron oxidation reactions with:

Protein-SH + N_2O_3 -----------> Protein-SNO + HNO_2 <u>Nitrosation</u>
Which results in S-nitrosothiols

                  peroxidase
Protein-SH + H_2O_2 -------------> Protein-SOH + H_2O <u>LP-complex</u>
Resulting in sulfenic acids.   (163,164)

In the progression from nitrosative to oxidative stress an increasing progression develops involving S-nitrosothiol, SNO, sulfenic acid, SOH, disulfide bonds, -S-S-, sulfinic acid, SO_2H, to sulfonic acid, SO_3H with the first three stages being reversible and the last two being irreversible.(165).

This manuscript, although a review of nitrosative stress due to reactive nitrogen species, (RNS), NO^-, NO^+, NO^-, N_2O_3, or RSNOs ONSCN, GSNO…and their impact on bacteriostasis will include and draw inferences from oxidative stress by reactive oxygen species, (ROS), O_2, H_2O_2, OH^-, or O_2^-. This is possible as nitrosation is a form of oxidative modification of reactive sulfhydryl groups.

The oxidating agent in the LP-complex has been identified as $OSCN^-$ as opposed to ONSCN formed from HNO_2/N_2O_3 in the nitrosative process.(166,167).

SCN^- + H_2O_2 -------------> $OSCN^-$ + H_2O lactoperoxidase

Thiocyanate serves as a cofactor and catalyst in both reactions as H_2O_2 and HNO_2 can directly inhibit bacteria but only at larger concentrations than in its presence.(168).

Although the LP-complex in theory should function as an antimicrobial system in vivo studies have resulted in mixed results. Numerous studies have failed to show an impact of the LP-complex on caries, possibly due to inadequate concentrations of H_2O_2 in saliva. (169-172). In addition in one study the mean salivary $OSCN^-$ concentrations were greater for smokers due to increased levels of thiocyanate in their saliva. This is in conflict with the fact that smokers have a higher caries rate than non-smokers.(173).

The bacteriostatic and bactericidal properties of the LP-complex will be illustrated in order to describe the effect of oxidative stress on lactic acid bacteria. Nitrosative stress involving N_2O_3, N_2O_4, GSNO, and ONSCN will proceed by a similar pathway, involving inactivation of glycolytic enzymes and glucose transport systems through interactions with reactive sulfhydryl residues. The analogous oxidation process has been utilized as this has provided addition data on the inhibitory effect of these systems.

Glycolytic enzyme inhibition through the oxidation of sulfhydryl groups have been identified in a number of studies. These enzymes have included aldolase, glyceraldehyde-3-phosphate dehydrogenase, GAPDH, and lactate dehydrogenase, LDH. Included is the inhibition of glucose

transport into the cell by the PEP-dependent phosphotransferase system, PTS, and the Proton Motive Force, PMF system.(174).

Of the glycolytic enzymes GAPDH is preferentially inactivated due to the low pKa, 5.5, of its reactive cysteine residue, Cys-149. As a result this reactive cysteine resides as a thiolate anion,(Cys-S$^-$), at physiologic pH and is more reactive to the nitrosonium ion, NO+.(175-177). GAPDH is 100 times more susceptible to inactivation compared with LDH.(178).

C. Schachtele has demonstrated in S. mutans that the membrane bound Enzymes II of the PTS is susceptible to N-ethylmaleimide,NEM, p-Chloromercuribenzoic acid, pCMB, and iodoacetic acid, IAA, inactivation, all sulfhydryl oxidizing agents.(179). In addition studies have shown a loss of the proton motive force in the PMF, possible due to an increase in cell wall permeability with efflux of K+ when bacteria are exposed to acidified nitrite or NEM.(180,181). OSCN $^-$ generated by the LP-complex also inactivates the PMF uptake of glucose.(182).

In a study of oxidative stress induced by H_2O_2 on S. mutans, without the other constituents of the LP-complex, the main site of inhibition was the glycolytic process. The most sensitive glycolytic enzyme was GAPDH with an IC50 of 2.2 mmol/L after 15 min. The next most prevalent site of inhibition was the PEP-dependent PTS with an IC50 of 10 mmol/L. Aldolase was not inhibited at bacteriostatic concentrations.(183).

Another bacteria with connections to the dairy process is Strep. agalactiae. When exposed to the LP-complex there was a strong inhibition of the transport of glucose into the cells with a complete inhibition of glycolysis. The inhibition of the PEP-dependent phosphotransferase system was deemed responsible for the lack of glucose transport. Glycolytic inhibition was expressed as a decrease in lactic acid production from 0.338 umol in controls to 0.099 in LP-complex exposed cells. The addition of dithiothreitol,DTT, a reducing agent, reversed the production to 0.402 umol.(184,185).

Hoogendoorn et al. demonstrated the effectiveness of the LP-complex against S. mutans as reflected in a decrease in acid production. The controls were able to decrease the pH from 6.8 to 5.9. The S. mutans cells remained at the 6.8 pH after inhibition with the LP-complex and the inhibition was reversed by glutathione, a low molecular weight thiol.(186).

In a study of the LP-complex inhibitory impact on S. mutans, S. salivarius, S. sanguis, and S. mitis GAPDH was identified as the glycolytic enzyme most affected. Intermediates in the pathway preceding GAPDH, glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bis-phosphate increased significantly. In addition intermediates following GAPDH, 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate, PEP decreased. Inhibition of PEP could in turn affect the transport of glucose by the PEP- dependant PTS.(187).

The process of glycolytic inhibition and glucose transport introduces an important topic of consideration. As enzymes associated with glycolysis are cytoplasmic we must consider how oxidative agents can transverse the cell membrane. Being a phospholipid the cell membrane is considered impermeable to charged particles. The pKa of OSCN$^-$ is 5.3 and it has been shown that at low pH it is in acid-base equilibrium with hypothiocyanous acid, HOSCN, which being uncharged may penetrate the cell membrane.(188). In both systems the pH plays a critical role. In the nitrate system the pH is critical for the production of nitrous acid from nitrite. Castellani and Niven found that the bacteriostatic effect of nitrite on a variety of bacteria increased 10 fold when the pH dropped one unit due to the increased production of nitrous acid.(189). It was demonstrated that HNO_2 may rapidly diffuse across the cell membrane into the cell and this occurred even at a pH of 7.(HNO_2 pKa = 3.14). In the cytoplasm nitrosative stress may also then result from N_2O_3 which is in equilibrium with HNO_2.(190). Although it is doubtful that RSNOs such as NOSCN may transverse cell membranes GSNO and other S-nitrosothiols were able to exert intracellular activity in epithelial cells, possibly through the release of free NO which is able to permeate the membrane. On the intracellular level NO$^.$ could auto-oxidize to N_2O_3. NOSCN may also have an effect on extracellular sugar transport systems.(191,192).

In Vitro Acidified Nitrite Studies

   We will next review literature based on nitrates bacteriostatic effect, information originating from food sciences, gastroenterology, pulmonary research, and the dental sciences.
   Sodium nitrite has been used as a food preservative for centuries. A complete review of nitrites antimicrobial activity and use in the food sciences was compiled by B. Tompkin.(193). Acidified nitrite has been shown to be effective against many food borne pathogens. Tarr et al.(1940), determined that sodium nitrite was bacteriostatic in as low a concentration as 0.02% at a pH of 6 but showed no inhibitory activity at pH7. This was the first study to demonstrate the dependence of nitrites bacteriostatic activity on pH.(194). Later studies proved sodium nitrites inhibitory activity against the food borne pathogen Clostridium perfringens and again the dependence on the pH. The concentration of nitrite required for inhibition of growth decreased as the pH increased, 3.8 to 15 times more sodium nitrite was required at a pH of 7.2 versus 6.3.(195). The inhibition was demonstrated by O'Leary and Solberg to be due to nitrites interaction with the sulfhydryl groups on glycolytic enzymes. There was a 91% decrease in the concentration of cystolic free sulfhydryl groups with a complete loss of GAPDH activity and a 67% decrease in aldolase activity.(196).
   Nitric oxide, $NO^.$, reacts with heme pigments to give cured meats their characteristic reddish color. Sodium nitrite is added to sausage in a fermentative process that involves the lactic acid bacteria Pediococcus cerevisiae. With the addition of sugar the lactic acid produced by the bacteria converts nitrite to nitrous acid. One role of the nitrous acid is to provide free $NO^.$ which produces the pink pigment in processed meats.(197).
   It was thought that this same agent, $NO^.$ contributed to nitrites antibacterial properties. It was determined by J. Shank et al. that $NO^.$ was inactive against Pseudomonas fluorescens, Staph. aureus, Strep. durans, Lactobacillus, and Clostridium. There was a significant increase in bactericidal and bacteriostatic activity when $NO^.$ was combined with NO_2. This can be explained by the equation:

$$NO^. + NO_2 \Longleftrightarrow N_2O_3 \quad \text{A nitrosating agent}$$
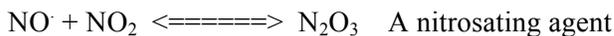
   In addition nitrous acid was demonstrated to have bactericidal and bacteriostatic effects.

$$2HNO_2 \Longleftrightarrow N_2O_3 + H_2O$$
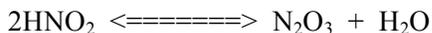
   The optimal pH was between 4.5 and 5.5 for bactericidal activity which became bacteriostatic between pH 5.6 and 5.8.(198).
   Pseudomonas aeruginosa is a pathogen that forms mucoid biofilms in cystic fibrosis, CF, patients. The biofilms are resistant to antibiotics but a novel treatment proposal is the use of aerosol nitrite. S. Yoon et al. report the killing of P. aeruginosa in an anaerobic biofilm analogous to plaque through exposure to acidified nitrite, 15 mM. There was no killing at pH 7.5 but nearly complete death of biofilm organisms at pH 6.5 implicating HNO_2 or its intermediate N_2O_3 as the active agent. The equilibrium concentration of HNO_2 would increase with increasing acidities, pKa = 3.14, and under two conditions that create a similar HNO_2 concentration, 10 uM, (pH 6.5 and 15 mM or pH 5.5 and 1.5 mM NO_2^-), killing was the same.(199). This demonstrates the antilog relationship of pH to nitrite concentration. Conditions of pH 5.5 and 1.5 mM NO_2^- replicate physiological conditions in the oral cavity.
   A study was conducted by Fite et al. on the effects of ascorbic acid, glutathione, thiocyanate, and iodide on the antimicrobial activity of acidified nitrite against the G.I. tract pathogen Yersinia enterocolitica at pH 2. Thiocyanate in concentrations from 0 to 500 uM was added to acidifed nitrite, pH2, in concentrations from 0 to 1000 uM. There was a decrease in the Minimum Bactericidal Concentration, MBC,(> 99.99% of cells killed), at increasing thiocyanate

concentrations and also iodide concentrations. Thiocyanate proved to be a more effective antimicrobial cofactor than iodide at equimolar concentrations. It was thought that ascorbic acid and glutathione, both being reducing agents, would potentiate the antimicrobial effect through the reduction of nitrous acid to NO.

$$2HNO_2 + \text{ascorbic acid} \quad \Longleftarrow\Longrightarrow \quad 2NO^. + 2H_2O + \text{dehydroascorbic acid}$$
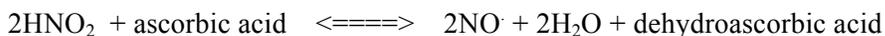
Instead the IC50,(50% inhibitory concentration), and the MBC increased for both agents. This indicated that NO. was not the active antimicrobial agent. Y. Bayindir determined that subjects with higher DMFT displayed greater concentrations of NO., more so in plaque than saliva. This would be in conflict with the theory that nitric oxide is the bacteriostatic agent and might indicate a protective mechanism in the survival of cariogenic bacteria through the reduction of HNO_2 or nitrosating agents to NO. by reducing agents/antioxidants in saliva.(200). Studies have shown a correlation between an increase in antioxidant/reducing capacity in saliva and increased caries rates.(201-205).

   D.L.H. Williams analyzed the decomposition of ONSCN in the presence of ascorbic acid. At low acidity, 0.5 M H_2SO_4, in the presence of excess N-methyl-N-nitrosoaniline as a nitrosating agent with thiocyanate at salivary concentrations ONSCN was formed and decomposed by 2.2 X 10^{-3} M ascorbic acid. (206). Ascorbic acid also acts as an inhibitor of the nitrosation process by decomposing HNO_2 to NO. as demonstrated above.

   Glutathione appears to play a protective role as a reducing agent in situations of oxidative and nitrosative stress. It is the largest non-protein thiol and antioxidant constituent in bacteria and mammalian cells. As evidenced in many studies it has the ability to reverse oxidative/nitrosative inhibition of critical sulfhydryl groups in enzymes or act as a scavenger of nitrosating agents through transnitrosation.(207,208).

 ENZYME-SNO + GSH ----------> ENZYME-SH + GSNO

RSNO + GSH <=========> RSH + GSNO

   Generally GSH is found in higher concentrations in gram negative bacteria due to their ability to produce the tripeptide versus gram positive bacteria which must import it from the surrounding media. Exceptions to this occur with Strep. agalactiae and L. lactis which can produce GSH.(209).
Agents which can decompose S-nitrosothiols include photolytic lyse, transition metals, l.m.w. thiols, antioxidants such as ascorbic acid, and enzymes specific to this activity. These enzymes include glutathione peroxidase,(210), xanthine oxidase,(211), the thioredoxin system,(212), and GSH-dependent formaldehyde dehydrogenase, GS-FDH, also identified as alcohol dehydrogenase III, ADH III, also known as bacterial GSNO reductase, GSNOR. Of these systems Xanthine oxidase may offer the least protection due to the low affinity for the substrate. FDH/ADH is specific to GSNO and is conserved from bacteria to yeasts, plants and animals. As GSNO is in equilibrium with S-nitrosothiols the enzyme has an impact on intracellular enzyme nitrosated thiols.(213). In the thioredoxin system reducing equivalents are transferred from NADPH through thioredoxin reductase to thioredoxin which decomposes nitrosothiols. Lactic acid bacteria do not have the ability to produce glutathione and can only import it from their medium. The FDH/ADH and Trx systems have therefore been proposed as major contributors to protection from oxidative/nitrosative stress. Both systems not only act as GSNO scavengers but also can denitrosate nitrosothiols in proteins.(214 -216).

Acidified Nitrite and Oral Lactic Acid Bacteria

We will next review studies that analyze nitrites impact on oral lactic acid bacteria. Xia et al. conducted research on acidifies nitrates and nitrites effect on 6 oral pathogens, S. mutans, L. acidophilus, Porphyromonas gingivalis, Capnocytophaga gingivalis, Fusobacterium nucleatum, and Candida Albicans. Nitrite in concentrations from .5 to 10 mmol/l had an inhibitory effect on all bacteria, the effect increasing with acidity. Strep. mutans was highly sensitive with Lactobacillus being more resistant. Acidified nitrate had no inhibitory effect. As low a concentration as 2 mmol/L nitrite, inhibited the growth of Strep. mutans at a pH of 7. At a pH of 6, 1 mM of nitrite was required and at a pH of 5 only .5 mM was necessary. A pH of 4.5 and 2 mmol/L was required for the same response by L. acidophilus.(217). Normal salivary nitrite concentrations are .27 but rise to 1.2 mmol/L one hour following a high nitrate meal, reaching 1.8 mmol/L at two hours and maintaining this concentration for 5 hours.(218). Consumption of 100-200 grams of green leafy vegetables results in salivary nitrite concentrations of 1-2 mM. Consumption of a 100 g mixed salad has resulted in salivary levels of 1.19 mM NO_2^-, well above levels proven to be bacteriostatic with in vitro experiments.(219).

Mendez et al. demonstrated similar results in a study of acidified nitrites effect on S. mutans, Lactobacillus casei, and Actinomyces naeslundii. At pH 4.5 to 7 there was a dose dependent reduction in bacterial counts for all species with .2 mM showing bacteriostatic capabilities in S. mutans at all pH values. Not only viability but also recovery from nitrite exposure was dose and pH dependent.(220).

Radcliffe et al. conducted similar tests on S. mutans , A. naeslandii, and L. casei. They determined that there was a minimal drop in the pH of the acid produced by the bacteria at .2 or 2.0 mM nitrite but a significant reduction in acid production at 20 and 200 mM. This larger concentration of nitrite, a level in excess of physiologic levels, necessary in this study versus studies previously discussed may be related to the addition of 1.0% m/v excess glucose in the growth medium. This may have allowed the increased production of NADPH by the Pentose Phosphate Pathway and used as a reducing cofactor in the Trx system.(221).

These studies demonstrate the antimicrobial capabilities of acidified nitrite on food borne pathogens, G.I. tract pathogens, and cariogenic bacteria. They substantiate the possible baseline capabilities of acidified nitrite which can be potentiated with the addition of thiocyanate in saliva.

Epidemiologic Surveys

We will examine data, some presented as a meta-analysis from epidemiologic surveys. The first two studies will compare green leafy vegetable consumption on a continent and countrywide basis with caries prevalence. Another two studies will compare nitrate ingestion derived from water supplies.

World Health Organization Surveys

As already stated approximately 85% of ingested nitrates in the diet are derived from vegetables. Those vegetables containing nitrate have been categorized into 5 groups by Corre and Breimer. Those vegetables in Group 5, with the highest nitrate content include beets, celery, lettuce, spinach, and radishes with greater than 2500 mg NO_3^-/kg. The description of green leafy vegetables is generally given to those plants with the greatest nitrate content. Although all these vegetables are being grouped according to predetermined mean values for nitrate concentrations the ranges of these figures vary widely. For instance the nitrate content of spinach was shown to vary from 2 to 6700 mg NO_3^-/kg in samples.(222).

The WHO developed a food monitoring program known as GEMS/Food. In 2008 a report from the European Food Safely Authority, EFSA, details nitrate consumption in a number of European and Eurasian countries in 2006.(84). In Table 9 countries are divided into clusters based upon common dietary patterns, this report deals with estimated vegetable consumption. Countries are

categorized into 4 clusters, B, D, E, and F, based on nitrate consumption, B having the largest consumption, F being the least. Besides each country is either an H,(high), L,(low), or ND,(no data). This information is derived from the WHO Global Oral Health Data Bank, WHO Oral Health Country/ Area Profile Programme and scientific reports. It reflects the oral health defined as DMFT for the listed countries with :

Low   5.0 - 13.9 DMFT

High   > 13.9   DMFT

Table 9

Cluster B		Cluster D		Cluster E		Cluster F	
Cypress	L	Albania	L	Austria	H	Estonia	H
Greece	H	Armenia	L	Belgium	H	Finland	H
Israel	L	Azerbaijan	ND	Croatia	H	Iceland	H
Italy	L	Belarus	L	Czech Rep.	H	Latvia	H
Lebanon	H	Bosnia/Herzegovina	H	Denmark	H	Lithuania	ND
Portugal	L	Bulgaria	H	France	H	Norway	H
Spain	L	Georgia	L	Germany	H	Sweden	H
Turkey	L	Iran	H	Hungary	H		
UAE	L	Kazakhstan	L	Ireland	H		
		Kyrgyzstan	ND	Luxembourg	H		
		Moldovia	L	Malta	L		
		Romania	L	Netherlands	H		
		Russia	L	Poland	H		
		Serbia/Montenegro	H	Slovakia	H		
		Tajikistan	ND	Slovenia	H		
		Macedonia	H	Switzerland	H		
		Turkmenistan	L	UK	H		
		Ukraine	L				
		Uzbekistan	L				
High	22.20%		31.30%		94.10%		100%
Low	77.80%		68.70%		5.90%		0%
Nitrate Consumption	204 mg/day		106 mg/day		98 mg/day		78 mg/day

   The data is for 35 to 44 year olds in 2004.(223). There is a pattern with the highest DMFT category being correlated with the lowest nitrate consumption and conversely the low DMFT category being associated with the highest nitrate consumption. The data presents a case for nitrates having a major impact on caries rates. Table 9 also shows the nitrate levels for each cluster group. This data is based on a concentration of 392 mg NO_3^-/kg for a diet consisting of a variety of vegetables. We see that nitrate consumption increases to 204 mg/day nitrate for Cluster B. Cluster D has an estimated nitrate intake of 106 mg/day, Cluster E is 98 and Cluster F is 78 mg/day. Cluster B has the lowest rate of caries indicating a level of nitrate where the cariostatic benefit of nitrate becomes most apparent.

Cluster D represents the reported average nitrate intake estimates for the U.S., 106 mg/d.(75). At this level we see an increase in nitrates impact on caries. The reason for the dramatic decrease between Cluster E and D may be due to nitrites antimicrobial activity being influenced by second order, exponential factors. Intake of 5.24 mM, 324 mg nitrate, results in salivary nitrite levels of 1.3 mM. There is approximately a 20 mg/L,(.44 mM), rise in salivary nitrite for every 100 mg nitrate ingested.(224).

Another factor of vegetable consumption is its possible relationship to socioeconomic status, SES. Dental caries has been recognized to be predicted by very few factors. Among them are previous dental treatment and SES, studies have shown an inverse relationship between SES and dental caries.(225-229). In a study by D. Forman et al. it was determined that those in the highest SES, class I and II, in the UK had a higher level of both nitrate and nitrite in their saliva than those in class III, which in turn had a higher level than those in class IV and V, the lowest SES class. This trend held for three different regions examined in the study and reflects a higher rate of vegetable consumption by higher SES individuals.(230).

It would seem logical to assume that the caries rates for vegetarians or vegans would be lower than for the general population due to a higher intake of vegetables. It is estimated that these individuals comprise 5 % of the population. A number of studies have shown a lower caries rate in these individuals.(231-234). Studies have also shown no difference in caries rates.(235). According to a UK study, MAFF 2000, the dietary nitrate intake of vegetarians and vegans does not exceed the ADI of 400 gr. vegetables and fruit requirement as cereals and nuts which are low in nitrate content tend to be substituted for the lack of protein products of animal origin.(84). Others have determined that vegetarians have a 2 to 4 fold higher average intake of nitrate than non-vegetarians.(236).

It is common knowledge that children and adolescents do not consume the recommended amounts of fruits and vegetables. This amounts to five servings of fruits and vegetables per person per day. Whereas adults consume 4.3 servings per day those ages 2-18 consume only 3.6 (0.10) with only .2 (0.02) servings out of a recommendation of 1 in the category of green/yellow vegetables. Only 12% of adolescents report eating three or more servings of vegetables with one being deep yellow or green in color. Household income was again identified as a positive influence in increasing the consumption of fruits and vegetables. Those not meeting vegetable intake requirements increased with age from 78.3% of 2-5 year olds, 83.8% of 6-11 years olds, to 89.5% of 12-18 year olds.(237-239).

In a study of children comparing salivary nitrite levels with caries, ages 6-12, it was determined that the control individuals had nitrite levels of 75.0 uM/L while those with Rampant Caries,RC, had levels of 32.4 uM/L. In a second age group, 71 months and younger, the control group had nitrite levels of 57.4 uM/L while those with Early Childhood Caries, ECC, had levels of 34.9 uM/L. In both groups of caries prone individuals the salivary nitrite levels were significantly lower than the control groups.(240).

M. Cingi et al. compared the salivary nitrite levels in 100 individuals by age a statistical difference was found in those 10-30, 12 +/- 2 mg/L versus those 31-60**,** 41 +/-5.(95). Increasing levels of salivary nitrate and nitrite with age were also found by Forman et al.,(139), and also in a study by Eisenbrand et al.(82).
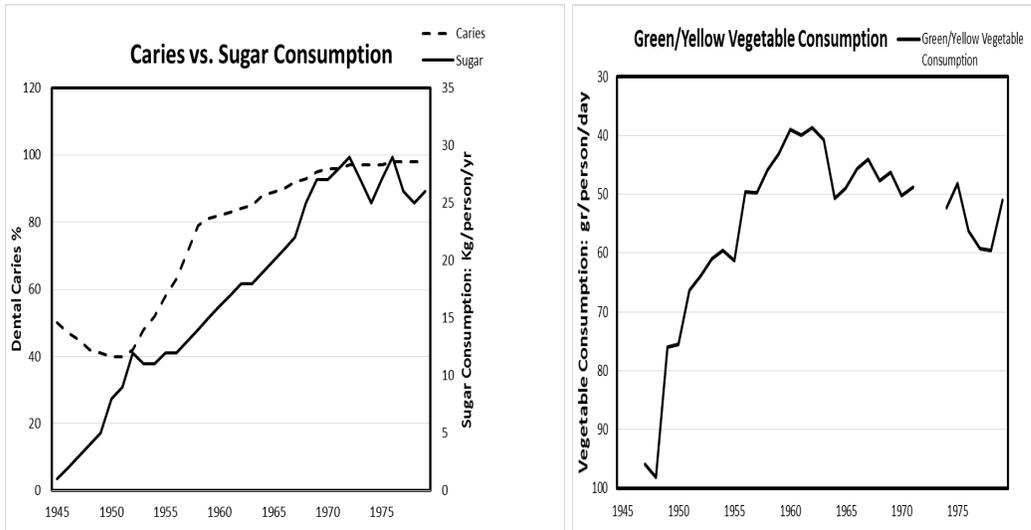
Japan

Japan was affected by the scarcity of sugar during WWII with a decrease in per capita consumption from 15 kg/yr/person in 1938 to nearly zero in 1946. There was a steady increase in sugar consumption from 1947 through 1973 with a gradual decline after this date. C.E. Renson

has charted sugar consumption versus a rapid rise in DMFT and demonstrates the close correlation of the two factors in Figure 1.

Figure 1 (Modified from fig. 4,ref. 241)   Figure 2

Of the 20 industrialized countries analyzed in the study only Japan showed an increase in caries rates after the war. This was attributed to the lack of fluoridation by Japan but others have demonstrated a decrease in caries rates in unfluoridated countries during the same time period.(241,242).   Following WWII there was a major change in the traditional Japanese diet with the introduction of western style foods. Some of this influence came from the necessity to provide food in an emergency situation for a starving nation following a devastating war. In 1954 a program was instituted, PL480, whereby surplus grains from the US were shipped to Japan.(243). Along with this change came a decrease in a traditional diet rich in vegetables and fish. Among those vegetables were the green, leafy varieties, along with mushrooms and seaweed, all high in nitrate content. In a study by T. Sobko et al. the levels of plasma and salivary nitrates and nitrites were determined for a control diet typical of western style foods compared with a traditional Japanese diet. The western style diet included foods such as cornflakes, muesli, yogurt, and sandwiches. Salivary nitrate levels were 569 uM/L versus 199.7uM/L in the Japanese diet compared with the western style control diet and 134.2 uM/L nitrite versus 71.9 uM/L respectively. Although the Japanese diet has what might be considered a higher than normal nitrate concentration being 4 times the E.U. ADI for nitrates it reflects the Japanese peoples diet prior to WWII.(244). Following the war a National Nutrition Survey was undertaken in Japan and has been conducted every year since then. According to the survey sugar consumption has increased from .5 gr/day/person in 1946 to a high of 19.9 gr/day/person in 1971. Concurrently the consumption of green and yellow vegetables has decreased from a high of 98.2 gr/person/day following WWII to a low of 38.6 gr/person/day in 1962. This trend is charted in inverse relationship in Figure 2. Outliers for 1972 and 1973 have been excluded.(245). We may infer that nitrates cariostatic effect decreased from 1945 onward with the replacement of the traditional Japanese diet with western style foods consisting of grains and meat products. It is the influence of both the increased consumption of sugar and the reduction of green vegetables in the diet that may have contributed to the rapid rise in caries.

Chile

Folklore has it that a handful of nitrate crystals were thrown on some growing plants which resulted in remarkable growth. The Atacama Desert of northern Chile contains the world's only commercially mineable resources of nitrate. There were 171 commercial open-pit nitrate mines in northern Chile, most of which closed by the 1950's. The Tarapaca Region of Northern Chile had the greatest number of mines and nitrate production. The towns in the region were closely associated with the nitrate mines and their survival was directly tied to the nitrate industry. Nitrate is deposited in layers of mineral conglomerates of salts called caliche. It forms layers from 1 to 6 feet thick near the surface so it is recovered by open-pit mining.(246).

Despite the arid nature of northern Chile water supplies tends to lie very close to the surface throughout the nitrate fields.(247). Drinking water will be the main source of nitrate exposure as vegetables have to be imported to the region and are considered a luxury. They are difficult to grow not only due to the arid climate but also the high salt content of the soil.(248). Minimal data exists on nitrate levels in water supplies in northern Chile.(249). Studies have been conducted on arsenic, perchlorate, and cyanide levels in drinking waters but nitrates have traditionally not been considered a pollutant or health concern until recently, especially in a region which prospered from the proceeds of nitrate mining. A study by R. Armijo in 1981 examines nitrate exposure as it relates to stomach cancer rates in various regions of Chile. Nitrate levels were analyzed in the urine of 11-13 year olds from 4 regions of Chile. Those children from a city in the north, Antofagasta, had urinary nitrate levels higher than those of the central cities of Chillan and Linares or those from the city of Punta Arenas in the south.(250).

In 1945 a report was issued by the Chilean Ministry of Health. It details caries experience in 37,051 12 to 14 year olds and indicates an influence of geographical location on caries rates. Chile lends itself to geographical surveys. It is 2,625 miles long with an average width of less than 100 miles, isolated by the Andes Mountains on the east and the Pacific Ocean on the west. Chile was divided into three regions for the study. The northern region includes the provinces from Tarapaca to Coquimbo, the central from Aconcagua to Valdivia, and the southern from Osorno to Magallanes. The northern children had 46% less caries experience compared with the central region, 57% less compared with the southern region. In 1960 a nutritional survey of Chile was conducted that included oral health. In a comparison of 902 military personnel the caries recorded as DMF were again lower for those individuals originating from the northern region as defined earlier, 8.9. The rate of caries was similar and higher in the central, 12.0, and southern regions, 11.1. A study conducted in the same survey with 665 women participants from the three regions found significantly less caries rates for those of the northern region, 9.9, than those of the central, 13.9, or southern regions, 11.9. Sugar intake was assessed and consumption/person/day was highest in the northern region with 61 gm/day versus 47 gm/day in the central region and 37 gm in the south. In 1960 a follow-up study was conducted involving 12 to 14 year olds involving 104 children. Again children from the north had a 59.1% lower DMF than the central and southern regions.(251).

Oregon

The Cascade Mountain Range runs in a north to south direction and divides Oregon into a western one-third and an eastern two-thirds. This mountain chain is the dominant factor in creating diverse and differing climactic conditions in the two regions of the state. Warm moist air off the Pacific Ocean is blocked by the Cascade Mountains resulting in an increased rainfall in the western portion of the state. The semi-arid climate and geography of Oregon adapts it well to agricultural practices in the eastern region. An additional region, the Willamette Valley, lies in western Oregon between a coastal range of mountains and Cascades and is a major agricultural area in the state. Generally private wells provide drinking water in the eastern region with community water supplies in the western region as this is where a majority of the urban centers are located. It has been determined that 22% of domestic wells exceed the U.S. MCL of 45mg/L

nitrate while only 5% of public water supplies exceed this limit.(252,253).

In a study by C. Chilvers et al. it was determined that when the waterborne nitrate concentration is below the EU limit of 50 mg/L nitrate, 30% of ingested nitrate comes from water. When the concentration increases to a level between 50 and 100 mg/L, 70% of daily intake is from water. Above 100 mg/L, 80% of ingested nitrate is waterborne.(254). Due to an increased concentration of agriculture and farming in the eastern region there are increased concentrations of nitrates in their drinking water. A number of studies have demonstrated that groundwater nitrate contamination is directly linked with agricultural and farming practices.(255-260). In addition the increased rainfall in the western regions contributes to the dilution of nitrates as they are leached into the groundwater.(77). The largest anthropogenic source is the application of fertilizers in addition to the agricultural process of ploughing fields and allowing them to lay fallow, ploughed but unseeded, following this activity. According to the nitrogen cycle in soils nitrogenous materials from decaying matter are decomposed to amino acids by bacteria, protozoa, yeasts, fungi, etc. and then deaminated to ammonia. The ammonia is then oxidized to nitrite by the bacteria Nitrosomonas and then nitrate by Nitrobacter in the soil.

A major source of nitrates is in the form of fertilizer applications. Fertilizer use increased dramatically following WW II with the conversion of munitions factories to fertilizer production. It increased from 5 pounds/acre in 1940 to 60 lbs/acre by 1960. By the 1980's fertilizer use per acre in this region exceeded 105 lb/acre and has stabilized at this point due to diminishing returns and better use, timing its application with growth cycles of the crops.(261). Still, the practice of overuse persists with some farmers applying more than recommended amounts. Winter wheat, a major crop in eastern Oregon is efficient in it uptake of nitrate but when too much fertilizer is applied or applied prior to precipitation there is increased leaching. Also, crops are rotated and many such as potatoes and sugar beets are not as effective at nitrate uptake, although the proper ordering of crops has been shown to decrease nitrate leaching.(262,263).

Surveys have been conducted to determine the population's exposure to nitrates in drinking water in eastern Oregon and the Willamette Valley in western Oregon. In 1996 a study of rural northeastern Oregon, the Lower Umatilla Basin of Umatilla and Morrow counties, determined that 25% of the wells had nitrate concentrations exceeding the US EPA limit of 10 mg/L nitrate-N,(45 mg/L NO_2). This resulted in 23% of the population being exposed to these concentrations. The region's main sources were identified as agriculture and farming, nitrogenous waste from a food processing plant, and septic systems. A 1990 study of another county in eastern Oregon, Malheur, identified 30% of the well water sites exceeding the 10 mg/L nitrate-N limit.(264). In 1993-95 a study was conducted on nitrates in the Willamette Valley. In this case 9% of the wells sampled, (n=70), had concentrations in excess of the EPA limit of 10 mg/L nitrate-N.(265). An additional study in 2000-2001 in the southern Willamette Valley found similar results with 7% of the wells exceeding the limit. The differences in nitrate concentrations assuming fertilizer use and applications being similar may result from two sources. The increased rainfall in the Willamette Valley will lead to a dilution of the leached nitrate. There may also be differences in soil quality, more porous soil lending itself to nitrate leaching.(266).

In 1959 a nutritional study of five western states included a caries rate survey in Oregon. Oregon was divided on a geographical basis into the eastern or 'central region' as defined by the study, the Willamette Valley, and the coastal region. The survey recognized a geographical influence on caries rates. Eastern Oregon exhibited a DMFT of 9.7. In the Willamette Valley the DMFT was 12.1 and on the coast it was 13.4. According to the model proposed for nitrates impact on caries this reflects the greater presence of agricultural practices in eastern Oregon with a minimal dilution of leached nitrate in the water supply, the region having annual precipitation of 12.8 inches at the time. Next in order is the Willamette Valley, again an agricultural region but with a greater dilution of leached nitrate with annual precipitation of 41.13 inches. Lastly we have the coastal region with minimal agricultural impact but maximum rainfall with annual precipitation of 70.23 inches per year and as much as 200 inches in some areas. This is reflected in the nitrate

concentrations in well water presented earlier. These differences could not be attributed to diet or fluoride in the drinking water.(267).

   Washington State has similar geographic and agricultural conditions and demonstrates the same distribution of caries rates. An empirical model developed by the USGS has determined that the southeast portion of the state will have higher nitrate concentrations in drinking water then the western portion of the state.(268). A study comparing bone density with caries rates found that those individuals that grew up in Yakama County located in the southeast region had lower caries rates then those from Snohomish County in the western portion of the state. According to the authors no apparent reason could be provided for the difference including fluoride in the water or dietary factors.(269).

Selenium

   The data from Oregon has in large part come from research on the connection of selenium and dental caries. It was believed that selenium contributed to the development of caries as higher concentrations were found in the soil and urine of those living in the higher caries rate regions, western Oregon. Extensive studies were conducted by D.M. Hadjimarkos from the University of Oregon. Selenium was implicated as being a causative factor in dental caries as the result of two studies done in the 1930's. It was noted that individuals living in seleniferous regions of rural Nebraska, Wyoming, and South Dakota had higher rates of caries.(270). These were followed by studies on dietary selenium intake and caries rates in children as it was thought selenium had a detrimental effect on enamel formation.(271). Higher caries rates have also been positively related to higher selenium concentrations in the urine of 14 to 16 year olds in Oregon.(272,273). A study in Wyoming of 10 to 18 year olds related an increased or decreased caries rate to living in high or low selenium regions, those living in high selenium regions had a caries rate 41% greater than those in a low selenium region in spite of a higher fluoride concentration in the drinking water. The intake of selenium from drinking water is negligible, nearly all of its intake is from food sources. A study in Montana, Oregon, and South Dakota related caries positively to selenium in the soil and forage plants resulting in increased levels in milk. No justification for seleniums activity could be provided although some thought it interacted with the enamel matrix inhibiting mineralization.(270,274). "Bad teeth" are one of the common symptoms seen in selenium toxicity.(275).

     Selenium (Se) is an essential microelement for human health. It is incorporated into human selenoprotein enzymes such as glutathione peroxidase (GPx), 5'-iodothyronine deiodinase (ID), and thioredoxin reductase (TR). There is an explanation for seleniums impact on caries rates in the Nitrate/Caries Model.

   Selenium in saliva has been shown to be closely associated with glutathione peroxidase, GPx. 81% of salivary Se is bound to GPx with another 19% bound to salivary proteins or enzymes. This characteristic is unique to saliva as other body fluids such as erythrocytes,(66%), plasma,(6%), and breast milk,(23%), show lower levels of GPx bound Se.(276).

   There are agents which can reverse the nitrosation of the sulfhydryl group. One of these is glutathione peroxidase which has been shown to decompose S-nitrosothiols.(210). Following the decomposition there is a release of nitric oxide, NO^-. GPx could also be a factor in resistance to nitrosative stress due to its ability to decompose RSNO's in saliva. Incubation of GSNO with GPx for 1 minute results in a 48.5% decrease in GSNO concentrations with the liberation of free NO^-. This same reaction was seen with CysNO.(277). When selenium was incorporated into the diets of Wistar rats there was an inhibition of the endogenous nitrosative process. Nitrosoproline, NPRO synthesis in the stomachs in the presence of nitrite and proline without selenium supplementation was 581.2 +/- 113.3 mg/kg body weight. With organic dietary Se supplementation there was a 54.5% decrease in NPRO production. Likewise without supplementation the addition of nitrite and diethylamine resulted in 29.8 +/- 3.0 mg/kg body

weight N-diethylamine, NDEA production and a 54.7% reduction with selenium supplementation.(278).

  The four nutritional sources of Se include the inorganic sources selenite,$(SeO_3)^{2-}$ and selenate,$(SeO_4)^{2-}$ for animals and plants and the organic sources selenocysteine,(SeCys) and selenomethionine,(SeMet) for animals. They are metabolized to a common intermediate, selenide which is incorporated into seleno-proteins as a selenol group, RSeH.(279).

      Another seleno-compound that may be capable of decomposition is selenocyanate. As mentioned earlier in this paper $SeCN^-$ may be transported by the NIS in the same manner as SCN^- ,I^-, and NO_3^-. It has antagonistic capabilities similar to thiocyanates.(7).
In a case history when large concentrations of selenite,(2500 mg over seven days), were administered to a patient they developed a severe case of hypothyroidism with iodide deficiency. $SeCN^-$ has a greater affinity for the NIS than SCN^-.(280). In toxic doses selenium may function not only in the role of decomposing nitrosocompounds but also as a goitrogen in the form of $SeCN^-$.(282). As mentioned earlier selenium is incorporated into 5'-iodotyronine iododinase which is responsible for the conversion of T4 to T3. Individuals, especially the elderly with low selenium status display a reduced T3/T4 ratio with selenium supplementation reversing the impairment. This demonstrates again how selenium status has an effect on selenoenzymes in the body.(282-284).

  $SeCN^-$ may react with free thiols, RSH, in saliva to produce a selenol, RSeH.

RSH + $(SeCN)_2$ <=======> RSSeCN + CNSeH
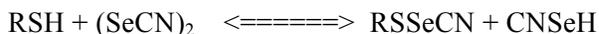
    The selenol produced from this reaction can then react with an S-nitrosothiol resulting in the release of $NO^.$ and the formation of a disulfide bond.(285).

2RS-NO + CNSeH --------->   RS-SR + $2NO^.$

Molybdenum

  Ludwig, Healy, and Losee found a lower caries rate for the town of Napier versus Hastings, New Zealand. The average DMFT for 6-10 year olds was 2.86 versus 3.56 respectively. New Zealand has always suffered from acidic soil, pH 5-7, which has a tendency to bind with molybdenum limiting its uptake by plants.(286). The inland agricultural town of Napier demonstrated a non-existent molybdate content in vegetables which is the main source of the trace element whereas the coastal town of Hastings demonstrated a molybdate content in vegetables .(287). A study by R. Anderson in England found a DMFT of 4.59 in 12 year olds,(n=682), from an area described as a molybdenum area versus 5.71 from a control area.(288). The possible mechanisms involved in molybdenums impact have usually involved the incorporation of the element into the enamel matrix in a manner similar to fluoride.(289,290).
  Molybdenum is required as a cofactor by over 40 different, mostly bacterial, enzymes. It is an important cofactor in two enzymes responsible for anaerobic reduction of nitrate to nitrite, formate dehydrogenase and nitrate reductase.(291). Molybdate, MoO_4^{2-}, is the cofactor, the soluble form of molybdenum available to plants and bacteria, that is combined with a pterin compound to form molybdopterin.(292). Formate is an efficient electron donor through the activity of formate dehydrogenase N.(293). Molybdate is required at a concentration of at least 10^{-6} M for nitrate reductase and the enzyme is not produced in its absence.(294).

Conclusion

If we are to believe that nitrate has an impact on caries rates than there are a number of factors that have an effect on this process. First and foremost is the ingestion of the nitrate itself, generally through a diet high in green, leafy vegetables. Added to this is the variable factors of absorption rates in the G.I. tract, concentration by the NIS, and conversion of nitrate to nitrite in the mouth. Additional factors include the catalytic effect of thiocyanate in the process and the particular bacteria impacted by the antimicrobial nitrosative process. Many of these factors are difficult to quantify. J. Dwyer et al. and H. Smiciklas-Wright state that food questionnaires used to gauge nitrate intake are inaccurate or at the least compromised.(295,296). Also due to the dependence of nitrate and nitrite concentrations in saliva on length of time after ingestion P. Packer et al. feels the only accurate measurement is a 24 hour urine analysis.(297). In spite of this nitrate or nitrite levels in saliva may serve as useful markers for an individuals predisposition for caries. This paper has also proposed as an alternative the use of cationic amino acids in saliva as a marker not only for nitrate ingestion but for caries rates as a whole. This proposal is based not only on nitrates impact on caries rates but also the catalytic impact of thiocyanate and to a lesser degree iodide levels all of which are reflected in cationic amino acid concentrations in saliva. It may be that due to a latency period of the cationic amino acids in the salivary gland their concentration in saliva may be less variable than nitrate/nitrite levels. Of course additional studies would be necessary to verify this concept.

   Because the sodium/iodide symporter is instrumental in the transport of nitrate and thiocyanate from serum to saliva any factor that affects it will also effect caries rates. A connection between hormonal inhibition in females has been made as well as the goitrogenic activity of lithium and elevated thiocyanate levels in smokers. Disease processes such as chronic renal failure have demonstrated that higher nitrate/nitrite levels in saliva may be reflected in decreased caries rates. Sjogrens Syndrome and radiation therapy have given insight into how decreased nitrate/nitrite levels in the context of decreased salivary flow may contribute to caries.

   An effort has been made to demonstrate how nitrate may have a bacteriostatic effect through the process of nitrosation resulting in oxidative/nitrosative stress. It has been demonstrated that this process may not only be detrimental to the growth and survival of the bacteria but may also specifically impact acid production through inhibition of glycolytic enzymes and sugar transport systems. Liberty has been taken in relying on studies from such varied fields as the food sciences and gastroenterology but much of the research in nitrosative reactions has been conducted outside the field of dental research.

   It has been documented that as low a nitrite concentration as 0.2 mM is bacteriostatic towards S. mutans. This concentration in saliva is easily obtainable with the ingestion of a 100 gr. mixed green salad. In addition a daily intake of 106 mg of nitrate appears to impart a beneficial effect on caries reduction. This may result in an estimated nitrite concentration of .44 mM in saliva which due to its half life may remain above 0.2 mM for up to 8 hours. Unfortunately due to the variability of nitrate in vegetables and drinking water it is difficult to quantify nitrate ingestion. What can be stated is that green leafy vegetables provide some protection against caries.

   As stated early in the paper much of the research on nitrosation has evolved from a concern that N-nitrosamines may cause cancer. Although over 300 of these agents have been identified as carcinogens in rat studies none have been directly implicated in human studies. In a comprehensive review of nitrate and cancer D. Forman comes to the conclusion that a cause and effect association has not been demonstrated.(298). Numerous studies have come to the conclusion that there is inconclusive evidence for a harmful effect from nitrates in terms of carcinogenesis in a normal diet.(146,299,300). In fact dietary levels of nitrate extend from 174 to 1222 mg in the DASH, Dietary Approaches to Stop Hypertension, diet which is recommended by the American Heart Association.(301). This paper is not advocating a dramatic increase in nitrate intake, as small a daily intake as 106 mg appears to have a positive impact on decreasing caries rates. This computes as the maximum recommended nitrate ingestion for a 63 lb. individual based upon the European Union A.D.I. of 0-3.7 mg/kg/d. A 121 lb. individual could likewise

consume enough nitrate to safely attain the highest level of 204 mg/d found in Cluster B. In order to localize the cariostatic activity it may be that a dentifrice with nitrite and thiocyanate may be beneficial in reducing dental caries.

The conclusion that nitrates may have a role in the caries process is not to detract from sugars major impact. It may be that there is a balance between the deleterious effects of sugar balanced with a protective effect imparted by nitrates in the diet.

REFERENCES

1. De la Vieja, A, Dohan, O, Levy, O, and Carrasco, N, Physiol. Rev. 2000;80(3): 1083-1105
2. Levy O, De la Vieja A, Carrasco N, J. Bioenerg. Biomembr. 1998;30(2),195-206
3. Spitzweg C, Heufelder A, Eur. J. Endocrinol. 1998:138(4);374-375
4. Dohan O, De la Vieja A, Paroder V, Riedel C, Artani M, Reed M, Ginter C, Carrasco N, Endocr. Rev. 2003;24(1):48-77
5. Carrasco N, Biochim. Biophys. Acta 1993:1154(1);65-82
6. Kaminsky S, Levy O, Salvador C, Dai G, Carrasco N, Soc. Gen. Physiol. Ser. 1993;48:251-262
7. Wolff J, Physiol. Rev. 1964:44(1);45-90
8. Harden R, Mason D, Buchanan W, J. Clin. Endocrinol. Metab. 1965;25:957-961
9. Myant N, Ann. NY. Acad. Sci. 1960;85:208-214
10. Brown-Grant K, Physiol. Rev. 1961;41(1):189-213
11. Ferguson M, Naimark A, Hildes J, Can. J. Biochem. Physiol. 1956;34(4):683-688
12. Matsuda A, Kosugi S, J. Clin. Endocrinol. Metab. 1997;82(12),3966-3971
13. VanWuyckhuyse B, Perinpanayagam H, Bevacqua R, Billings R, Bowen W, Tabak L, J. Dent. Res. 1995;74(2):686-690
14. Calandra J, Fosdick L, J. Dent. Res. 1947;26(4):303-308
15. McClure F, J. Nutr. 1958;65(4):619-631
16. McClure F, Folk J, Science 1955;122(3169):557-558
17. Vranic L, Granic P, Rajic Z, Acta. Stomatol. Croat. 1991;25:71-76
18. Giebel W, Striebel H, Breuninger H, Laryng. Rhinol. Otol. 1982;61:70-74
19. Lengeler J, Drews G, Schlegel H, Biology of the Prokaryotes, 1st ed. Thieme Stuttgart NY
20. Kanai Y, Yoshiki F, Ho Cha S, Segawa H, Chairoungdua A, Kim D, Matsuo H, Kim J, Miyamoto K, Takeda E, Endou H, J. Biol. Chem. 2000;275(27):20787-20793
21. Elano N, Deves R, Boyd A, J. Physiol. 1994;479(2):291-300
22. Deves R, Angelo S, J. Biol. Chem. 1996;271(50):32034-32039
23. Kavanaugh M, Biochem. 1993:32(22):5781-5785
24. Lauger P, Electogenic Ion Pumps. Distinguished Lecture Series of the Society of General Physiologists, Vol. 5, Sinauer Associates, Inc. Sunderland,MA
25. Bronner F, Kleinzeller A, Current Topics in Membranes and Transport, Vol. 9, Academic Press,NY 1977
26. O'Neill B, Magnolato D, Semenza G, Biochimica Et Biophysica Acta 1987;896: 263-274
27. Boyd C, Vatish M, Jpn. J. Physiol. 1994;44(Suppl. 2):S273-S280
28. Wapnir I, Van de Rijn M, Nowels K, Amenta P, Walton K, Montgomery K, Greco R, Dohan O, Carrasco N, J. Clin. Endocrinol. Metab. 2003;88(4):1880-1888
29. Jhiang S, Cho J, Ryu K, DeYoung B, Smanik P, McGaughy V, Fischer A, Mazzaferri E, Endocrin. 1998;139(10):4416-4419
30. Vayre L, Sabourin J, Caillou B, Ducreux M, Schlumberger M, Bidart J, Eur. J. Endocrinol. 1999;141:382-386
31. Burgen A, Int. Ser. Monogr. Oral Biol. 1994;3:197-215
32. Lindsay R, Catanzaro O, Ueha T, Hanson R, Am. J. Physiol. 1969;217(4):1025-1029
33. Lukacs J, Curr Anthropol. 2008;49(5):901-914
34. Haugejorden O, Community Dent. Oral Epidemiol. 1996;24:369-375
35. Lukacs J, Am. J. Hum. Biol. 2011;23,398-411
36. Hong-Ying W, Petersen P, Jin-You B, Bo-Xue Z, Int. Dent. J. 2002;52:283-290
37. Matthesen M, Barlum V, Aarslev I, Fejerskov O, Community Dent. Health 1990;7:123-133
38. Lukacs J, Largaespada L, Am. J. Hum. Biol. 2006;18:540-555
39. Sehested A, Juul A, Andersson A, Petersen J, Jensen T, Muller J, Skakkebaek N, J. Clin. Endocrinol. Metab. 2000;85(4):1634-1640
40. Angsusingh, K, Kenny F, Nankin H, Taylor F, J. Clin. Endocrinol. Metab. 1974;39(1):63-68
41. Andersson A, Juul A, Petersen J, Groome N, Skakkebaek N, J. Clin. Endocrinol. Metab. 1997;82(12):3976-3981
42. Loriaux D, Ruder H, Knab D, Lipsett M, J. Clin. Endocrinol. Metab. 1972;35(6): 887-891
43. DeHertogh R, Thomas K, Bietlot Y, VanDerHeyden I, Ferin J, J. Clin. Endocrinol. Metab. 1975;40(1):93-101
44. Christensen K, Gaist D, Jeune B, Vaupel J, Lancet 1998;352(9123):204
45. Halling A, Bengtsson C, Community Dent. Health 1989;6:39-45

46. Rakchanok N, Amporn D, Yoshida Y, Harun-Or-Rashid M, Sakamoto J, Nagoya J. Med. Sci. 2010;72:43-50

47. Turnbridge W, Evered D, Hall R, Appleton D, Brewis M, Clark F, Evans J, Young E, Bird T, Smith P, Clin. Endocrinol. 1977;7:481-493

48. VanderPump M, Tunbridge W, French J, Appleton D, Bates D, Clark F, Evans J, Hasan D, Rodgers H, Tunbridge F, Young E, Clin. Endocrinol.1995;43: 55-68

49. Lind P, Langsteger W, Molnar M, Gallowitsch H, Mikosch P, Gomez I, Thyroid 1998;8(12):1179-1183

50. Marine D, Surg. Gynecol. Obstet. 1917;25:272-275

51. Glinoer D, Lemone M, Thyroid 1992;2(1):65-70

52. Braverman L, Utiger R, The Thyroid, 7th ed.Lippincott-Raven, Philadelphia, NY

53. Furlanetto T, Nguyen L, Jameson L, Endocrinology 1999;140(12):5705-5711

54. Furlanetto T, Nunes R, Sopelsa A, Maciel R, Braz. J. Med. Biol. Res. 2001;34: 259-263

55. Robinson H, J. Dent. Res. 1948;27(1):113-122

56. Cortas N,Wakid N, Pharmacol. Toxicol. 1990;68:192-195

57. Tannenbaum S, Sinskey A, Weisman M, Bishop W, J. Natl. Cancer Inst. 1974;53(1):79-84

58. Schreiber F, Stief P, Gieseke A, Heisterkamp I, Verstraete W, DeBeer D, Stoodley P, BMC Biology 2010;8:24 p.1-11

59. Williams DLH, Nitrosation, Cambridge University Press, Cambridge, UK 1988

60. Ridd J, Q. Rev. Chem. Soc. 1961;15:418-441

61. Mirvish S, Toxicol. Appl. Pharmacol. 1975;31:325-351

62. Marquis R, J. Ind. Microbiol. 1995;15:198-207

63. Cole J, Biochem. Soc. Trans. 1977;5(4):1232-1239

64. VanHoute J, J. Dent. Res. 1994:73(3);672-681

65. Jensen M, Wefel J, Br. Dent. J. 1989;167:204-208

66. Tannenbaum S, Archer M, Wishnok J, Bishop W, J. Natl. Cancer Inst. 1978;60(2):251-253

67. Rao, G, N. Engl. J. Med. 1978:p202

68. Simon D, Mullins M, Jia L, Gaston B, Singel D, Stamler J, Proc. Natl. Acad. Sci. USA 1996;93:4736-4741

69. Stamler J, Singel D, Loscalzo J, Science 1992;258:1898-1902

70. O'Neill I, Ohshima H, IARC 1987;84:322-327

71. Park J, Biochem. Biophys. Res. Comm. 1988;152(2):916-920

72. Takahama U, Hirota S, Kawagishi S, Free Radic. Res. 2009;43(3):250-261

73. DaSilva G, Kennedy E, Dlugogorski B, J. Am. Chem. Soc. 2005;127:3664-3665

74. Licht W, Deen W, Carcinogenesis 1988;9(12):2227-2237

75. Gangolli S, Vanden Brandt P, Feron V, Janzowsky C, Koeman J, Speijers G, Spiegelhalder B, Walker R, Wishnok J, Environ. Toxicol. Pharmacol. 1994;292:1-38

76. Cammack R, Joannou C, Cui X, Martinez C, Maraj S, Hughes M, Biochimica et Biophysica Acta 1999;1441:475-488

77. Hill M, Nitrates and Nitrites in Food and Water, Woodhead Publishing Ltd., Cambridge, UK 1996

78. Van Velzen A, Sips A, Schothorst R, Lambers A, Meulenbelt J, Tocicol. Lett. 2008;181:177-181

79. Tannenbaum S, Weisman M, Fett D, Fd. Cosmet. Toxicol. 1976;14:549-552

80. Doel J, Hector M, Amirtham C, Al-Anzan L, Benjamin N, Allaker R, Eur. J. Oral Sci. 2004;112:424-428

81. Spiegelhalder B, Eisenbrand G, Preussmann R, Fd. Cosmet. Toxicol. 1976;14: 545-548

82. Walters C, Smith P, Fd. Cosmet. Toxicol. 1981;19:297-302

83. Eisenbrand G, Spiegelhalder B, Preussmann R, Oncology 1980;37:227-231

84. Nitrate in Vegetables: Scientific Opinion of the Panel on Contaminants in the Food chain. The EFSA Journal. 2008;689:1-79

85. Stephany R, Schuller P, IARC Sci. Publ. 1978;19:443-460

86. Tonacchera M, Pinchera A, Dimida A, Ferrarini E, Agretti P, Vitti P, Santini F, Crump K, Gibbs J, Thyroid 2004;14(12):1012-1019

87. Ermans A, Delanga F, VanderVelden M, Kinthaert J, Adv. Exp. Med. Biol. 1972;30:455-486

88. Saunders J, Himwich W, Am. J. Physiol. 1950;163(2):404-409

89. Haley N, Axelrad C, Tilton K, Am. J. Public Health 1983;73:1204-1207

90. Walker R, Fd. Cosmet. Toxicol. 1978;17:473-479

91. Luepker R, Pechacek T, Murray D, Johnson C, Hund F, Jacobs D, Am. J. Public Health 1981;71:1320-1324

92. Tenovuo J, Makinen K, J. Dent. Res. 1976;55(4):661-663

93. Vought R, London W, Metabolism 1965;14(6):699-707

94. Mirvish S, Reimers K, Kutler B, Chen S, Haorah J, Morris C, Grandjean A, Lyden E, Eur. J. Cancer Prev. 2000;9:335-342

95. Cingi M, Cingi C, Cingi E, Bull. Environ. Contam. Toxicol. 1992;48:83-88

96. Chow M, Peterson D, Oral Surg. Med. Oral Pathol. 1979;48(1):34-38

97. Jaffe E, Roberts G, Chantler C, Carter J, Br. Dent. J. 1986;160(1):18-20

98. Wolff A, Stark H, Sarnat H, Binderman I, Eisenstein B, Drukker A, Int. J. Pediatr. Nephrol. 1985;6(2):127-132

99. Peterson S, Woodhead J, Crall J, Pediatr. Res. 1985;19(8):796-799

100. Nowaiser A, Roberts G, Trompeter R, Wilson M, Lucas V, Pediatr. Nephrol. 2003;18:39-45

101. Nunn J, Sharp J, Lambert H, Plant N, Coulthard M, Pediatr. Nephrol. 2000;14:997-1001

102. Lucas V, Roberts G, Pediatr. Nephrol. 2005;20:1388-1394

103. Davidovich E, Schwarz Z, Davidovitch M, Eldelman E, Bimstein E, J. Clin. Periodontol. 2005;32:1076-1082

104. Nowaiser A, Lucas V, Wilson M, Roberts G, Trompeter R, Int. J. Paediatr. Dent. 2004;14:118-126

105. Tabak L, Pediatr. Dent. 2006;28:110-117

106. Nishimuta M, Takahashi H, Maruyama K, Ohsuka K, Nanbu A, Hara K, Yoshimura M, Am. J. Kidney Dis. 1998;31(5):809-817

107. Blicharz T, Rissin D, Bowden M, Hayman R, DiCesare C, Bhatia J, Grand-Pierre N, Siqueira W, Helmerhorst E, Loscalzo J, Oppenheim F, Walt D, Clin. Chem. 2008;54(9):1473-1480
108. Cailleux A, Subra J, Riberi P, Tuchais E, Premel-Cabic A, Allain P, J. Med. 1988;19(5&6):345-351
109. Koyama K, Yoshida A, Morozumi K, Fujinami T, Tanaka N, Nephrol. Dial. Transplant 1997;12:1622-1628
110. Fox P, Bowman S, Segal B, Vivino F, Murukutla N, Choueiri K, Ogale S, J. Am. Dent. Assoc. 2008;139(12):1592-1601
111. Baudet-Pommel M, Albuisson E, Kemeny J, Falvard F, Ristori J, Fraysse M, Sauvezie B, Oral Surg. Oral Med. Oral Pathol. 1994;78:181-186
112. Christensen L, Petersen P, Thorn J, Schiodt M, Acta. Odontol. Scand. 2001;59(3):116-120
113. Petersen A, Bardow A, Nauntofte B, BMC Clin. Pathol. 2005;5(4):1-13
114. Mahoney E, Spiegel J, Otolaryngol. Clin. N. Am. 2003;36:733-745
115. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Lohrs U, Virchows Arch. 1997;434:315-323
116. Atkinson J, Fox P, Clin. Geriatr. Med. 1992;8(3):488-511
117. Xia D, Deng D, Songlin W, J. Oral Pathol. Med. 2003;32:95-99
118. Granli T, Dahl R, Brodin P, Bockman O, Fd. Chem. Toxic. 1989;27(10):675-680
119. Harden R, Alexander W, Proc. R. Soc. Med. 1968;61(7):647-649
120. Zeldow B, J. D. Res. 1959;18(4):798-804
121. Xia D, Deng D, Wang S, J. Dent. Res. 2003;82(2):101-105
122. Stephen K, Robertson J, Harden R, Chisholm D, J. Lab. Clin. Med. 1973;81(2):219-229
123. HardenR, Mason D, Alexande, W, Quart. J. Exp. Physiol. 1966;51:130-135
124. Wang W, Chen Y, Lin L, Auris Nasus Larynx 2008;35:230-234
125. Konjhodzic-Prcic A, Ajanovic M, Smajkic N, Hasic-Brankovic L, Pesq. Bras. Odontoped. Clin. Integr. 2010;10(3):489-492
126. Pow E, McMillan A, Leung W, Kwong D, Wong M, Oral Diseases 2003;9:196-202
127. Dreizen S, Brown L, Daly T, Drane J, JDR 1977;56(2):99-104
128. Radfar L, Sirois D, Oral Surg. Oral Med. Oral Pathol. 2003;96(3):267-274
129. Jham B, Reis P, Miranda E, Lopes R, Carvalho A, Scheper M, Freire A, Clin. Oral Invest. 2008;12:19-24
130. Chen C, Ren F, Lu T, Friss T, He T, Zhang X, Jian Y, Arch. Oral Biol. 2010;55:613-620
131. Ludwick W, Massler M, J. Dent. Res. 1952;31(3):319-322
132. Ainamo J, Suom. Hammaslaak. Toim. 1971;67:87-94
133. Zitterbart P, Matranga L, Christen A, Park K, Potter R, Gen. Dent. 1990;38(6):426-431
134. Hirsch J, Livian G, Edward S, Noren J, Swed. Dent. J. 1991;15:117-123
135. Axelsson P, Paulander J, Lindhe J, J. Clin. Periodontol. 1998;25:297-305
136. Hegedus L, Karstrup S, Veiergang D, Jacobsen B, Skovsted L, Feldt-Rasmussen U, Clin. Endocrinol. 1985;22:287-292
137. Ladd K, Archer M, IARC 1984;57:811-817
138. Ladd K, Newmark H, Archer M, JNCI 1984;73:83-87
139. Forman D, Al-Dabbagh S, Doll R, Nature 1985;313:620-625
140. Aligne C, Moss M, Auinger P, Weitzman M, JAMA 2003;289(10):1258-1264
141. Shenkin J, Broffitt B, Levy S, Warren J, J. Public Health Dent. 2004;64(3):184-186
142. Tanaka K, Hanioka T, Miyake Y, Ojima M, Aoyama H, J. Public Health Dent. 2006;66(4):279-281
143. Krebs L, (2001) http://faculty.mckendree.edu/scholars/2001/krebs.htm
144. Markitziu A, Shani J, Avni J, Oral Surg. Oral Med. Oral Pathol. 1988;66:551-557
145. Friedlander A, Nichols D, Birch J, Spec. Care Dentist 1990;10(5):148-151
146. Beach W, Dental Cosmos 1908;50(5): 469-472
147. Zeldow B, J. Immunol. 1963;90:12-16
148. Dogon I, Kerr A, Amdur B, Arch. Oral. Biol 1962;7: 81-90
149. Jeon J, Rosalen P, Falsetta M, Koo H, Caries Res. 2011;45:243-263
150. Darout I, Albandar J, Skaug N, Raouf A, J. Clin. Periodontol. 2002;29:411-420
151. Almas K, Al-Zeid Z, J. Contemp. Dent. Pract. 2004;5(1):105-114
152. Al-Bagieh N, Weinberg E, Microbios. Letters 1988;39:143-151
153. Sofrata A, (2000) Salvadora Perscica (Miswak) Div. of Periodontol. Karolinska Institutet, Stockholm,Sweden
154. Sultana T, Porter N, Savage G, McNeil D, J. Agric. Food Chem. 2003;51:3586-3591
155. Masuda H, Inoue T, Kobayashi Y, In Oriental Foods and Herbs, 2003;Ch. 9,142-153 ISBN 9780841238411
156. Higdon J, Delage B, Williams D, Dashwood R, Pharmacol. Res. 2007;55(3):224-236
157. Morris S, Hansen N, J. Bacteriol. 1981;148(2):465-471
158. Klebanoff S, Luebke R, Exp. Biol. Med. 1965;118(2):483-486
159. Klebanoff S, Clem W, Luebke R, Biochim. Biophys. Acta 1966;117:63-72
160. Reiter B, Marshall V, Bjorck L, Rosen C, Infect. Immun. 1976;13(3):800-807
161. Thomas E, Aune T, Antimicrob. Agents Chemother. 1978;13(6):1006-1010
162. Klatt P, Lamas S, Eur. J. Biochem. 2000;267:4928-4944
163. Thomas J, Mallis R, Exp. Gerontol. 2001;36:1519-1526
164. Aune T, Thomas E, Biochemistry 1978;17(6):1005-1010
165. Hess D, Matsumoto A, Kim S, Marshall H, Stamler J, Nat. Rev. Mol. Cell Biol. 2005;6(2):150-166
166. Aune T, Thomas E, Eur. J. Biochem. 1977;80:209-214
167. Pruitt K, Mansson-Rahemtulla B, Tenovuo J, Archs. Oral Biol. 1983;28(6):517-525

168. Thomas E, Milligan T, Joyner R, Jefferson M, Infect. Immun.1994;62(2):529-535
169. Grahn E, Tenovuo J, Lehtonen O, Eerola E, Vilja P, Acta Odontol. Scand. 1988;46:67-74
170. Mandel J, Behrman J, Levy R, Weinstein D, J. Dent. Res. 1983;62(8):922-925
171. Hoogehdoorn H, The Effect of Lactoperoxidase-Thiocyanate-Hydrogen Peroxide on the Metabolism of Cariogenic Micro-organisms in Vitro and in the Oral Cavity, Doctor in Technical Sciences thesis, Mouton, Den-Haag, 1974
172. Lenander-Lumikari M, Loimaranta V, Adv. Dent. Res. 2000;14:40-47
173. Lamberts B, Pruitt K, Pederson E, Golding M, Caries Res. 1984;18:488-494
174. Yarbrough J, Rake J, Eagon R, Appl. Environ. Microbiol. 1980;39(4):831-834
175. Padgett C, Whorton R, Am. J. Physiol. 1995;269(3 Pt 1):C739-749
176. Zhou W, Han P, Jiangmei L, Zhang X, Huang B, Ruan H, Chen C, PLoS One Apr 2010:2;5(4):e10015. doi 10.1371/journal.pone.0010015
177. Talfournier F, Colloc'h N, Mornon J, Branlant G Eur. J. Biochem. 1998;252:447-457
178. Vedia L, McDonald B, Reep B, Brune B, DiSilvio M, Billiar T, Lapetina E, J. Biol. Chem. 1992;267(35):24929-24932
179. Schachtele C, JDR 1975;54(2):330-338
180. Kashani H, Nikzad H, Mobaseri S, Hoseini E, Life Sci. J. 2012;9(1):496-501
181. Meury J, Lebail S, Kepes A, Eur. J. Biochem. 1980;113:33-38
182. Reiter B, Ann. Rech. Vet. 1978;9(2):205-224
183. Baldeck J, Marquis R, Can. J. Microbiol. 2008;54:868-875
184. Mickelson M, J. Bacteriol. 1977:132(2);541-548
185. Mickelson M, Appl. Environ. Microbiol. 1979;38(5):821-826
186. Hoogendoorn H, Piessens J, Scholtes W, Stoddard L, Caries Res. 1977;11:77-84
187. Carlsson J, Iwami Y, Yamada T, Infect. Immun. 1983;40(1):70-80
188. Tenovuo J, Makinen K, Sievers G, Antimicrob. Agents Chemother. 1985;27(1):96-101
189. Castellani A, Niven C, Appl. Microbiol. 1955;3(3):154-159
190. Parsonage D, Greenfield J, Ferguson S, Biochim. Biophys. Acta 1985;807:81-95
191. Mathews W, Kerr S, J. Pharmacol. Exp. Ther. 1993;267(3):1529-1537
192. Ignarro L, Hypertension 1990;16(5):477-483
193. Tompkin R, Nitrite. In: Davidson P, Sofos J, Branen A, ed. Antimicrobials in Food 3rd ed. CRC Press 2005, p. 169-236
194. Tarr H, J. Fish. Res. Bd. Can. 1941;5(3):265-275
195. Riha W, Solberg M, J. Food Sci. 197540(3);439-442
196. O'Leary V, Solberg M, Appl. Environ. Microbiol. 1976;31(2):208-212
197. Laboratory Manual for the Food Microbiology Laboratory, (1998 ed.) Bacteriology/Food Science 324, Univ. Wis.-Madison. www.jlindquist.net/generalmicro/324sausage.html
198. Shank J, Silliker J, Harper R, Appl. Microbiol 1962;10(3):185-189
199. Yoon S, Coakley R, Lau G, Lymar S, Gaston B, Karabulut A, Hennigan R, Hwang S, Buettner G, Schurr M, Mortensen J, Burns J, Speert D, Boucher R, Hassett D, J. Clin. Invest. 2006;116:436-446
200. Bayindir Y, Polat M, Seven N, Caries Res. 2005;39:130-133
201. Tulunoglu O, Demirtas S, Tulunoglu I, Int. J. Paediatr. Dent. 2006;16:186-191
202. Kumar D, Pandey R, Agrawal D, Agrawal D, Int. J. Paediatr. Dent. 2011;21:459-464
203. Hegde A, Rai K, Padmanabhan V, J. Clin. Pediatr. Dent. 2009;33(3):231-234
204. Uberos J, Alarcon J, Penalver M, Molina-Carballo A, Ruiz M, Gonzalez E, Castejon J, Munoz-Hoyos A, Br. Dent. J. 2008;205(2),1-4
205. Hegde M, Kumari S, Moany A, Jathanna V, JIDA 2012;6(2):75-77
206. Williams D, Fd. Cosmet. Toxicol. 1978;16:365-367
207. Kullisaar T, Songisepp E, Aunapuu M, Kilk K, Arend A, Mikelsaar M, Rehema A, Zilmer M, Prikl. Biokhim. Mikrobiol. 2010;46(5):527-531
208. Hogg N, Annu. Rev. Pharmacol. Toxicol. 2002;42:585-600
209. Fahey R, Brown W, Adams W, Worsham M, J. Bacteriol. 1978;133(3):1126-1129
210. Hou Y, Guo Z, Li J, Wang P, Biochem. Biophys. Res. Commun. 1996;228:88-93
211. Trujillo M, Alvarez M, Peluffo G, Freeman B, J. Biol. Chem. 1998;273(14):7828-7834
212. Nikitovic D, Holmgren A, J. Biol. Chem. 1996;271(32):19180-19185
213. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler J, Nature 2001:410(6827):490-494
214. Benhar M, Forrester M, Stamler J, Nat. Rev. Mol. Cell Biol. 2009;10(10):721-732
215. Benhar M, Thompson J, Moseley M, Stamler J, Biochem. 2010;49(32):6963-6969
216. Foster M, Hess D, Stamler J, J. Trend Mol. Med. 2009;15(9):391-404
217. Xia D, Liu Y, Zhang C, Tang S, Wang S, Chin. Med. J. 2006;119(22):1904-1909
218. Pannala A, Mani A, Spencer J, Skinner V, Bruckdorfer K, Moore K, Rice-Evans C, Free Radic. Biol. Med. 2003;34(5):576-584
219. Peri L, Pietraforte D, Scorza G, Napolitano A, Fogliano V, Minetti M, Free Radic. Biol. Med. 2005;39:668-681
220. Silva Mendez S, Allaker R, Hardie J, Benjamin N, Oral Microbiol. Immunol. 1999;14:391-392
221. Radcliffe C, Akram N, Hurrell F, Drucker D, J. Dent. 2002;30:325-331
222. Corre W, Breimer T, Nitrate and Nitrite in Vegetables.Literature Survey No. 39, Centre for Agricultural Publishing and Documentation. Wageningen, The Netherlands.
223. Petersen P, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C, Bulletin of the World Health Organization 2005;83:661-669
224. Shapiro K, Hotchkiss J, Roe D, Fd. Chem. Toxic. 1991;29(11):751-755
225. Peres M, Peres K, Dornellas de Barros A, Victoria C, J. Epidemiol. Community Health 2007;61:141-145

226. Vargas C, Crall J, Schneider D, J. Am. Dent. Assoc. 1998;129(9):1229-1238
227. Meneghim M, Kowlowski F, Pereira A, Ambrosano G, Meneghim Z, Cien Saude Colet 2007;12(12):523-529
228. Rehman M, Mahmood N, Rehman B, J. Ayud Med. Coll. Abbottabad 2008;20(1):103-108
229. Reisine S, Psoter W, J. Dent. Educ. 2001;65(10):1009-1016
230. Forman D, Al-Dabbagh S, Doll R, IARC Sci. Publ. 1984;57:901-909
231. Linkosalo E, Proc. Finn. Dent. Soc. 1988;84(Suppl 8-9):1-77
232. Glass R, Hayden J, J. Dent. Child 1966;33(1):22-23
233. Donnelly C, Public Health Rep. 1961;76(3):209-212
234. Rahmatulla M, Gulle E. Community Dent. Oral Epideniol. 1990;18:277-278
235. Linkosalo E, Ohtonen S, Markkanen H, Karinpaa A, Kumpusalo E, Scand. J. Dent. Res. 1985;93:304-308
236. Mensinga T, Speijers G, Meulenbelt J, Toxicol. Rev. 2000;22(1):41-51
237. Krebs-Smith S, Cook A, Subar A, Cleveland L, Friday J, Kahle L, Arch. Pediatr. Med. 1996;150:81-86
238. Neumark-Sztainer D, Wall M, Perry C, Story M, Prev. Med. 2003;37:198-208
239. Lorson B, Melgar-Quinonez R, Taylor C, J. Am. Diet Assoc. 2009;109:474-478
240. Hegde A, Neekhra V, Shetty S, J. Clin. Pediatr. Dent. 2008;32(4):283-286
241. Renson C, Ann. Acad. Med. Singapore 1986;15(3):284-298
242. Health Effects: Tooth Decay Trends in Fluoridated vs. Unfluoridated Countries, Fluoride Action Network Health Effects http://www.fluoridealalert.org/health/teeth/caries/who-dmft.html
243. Selden M, The Other Japan, M.E. Sharpe, Armonk NY 1997
244. Sobko T, Marcus C, Govoni M, Kamiya S. Nitric Oxide 2010;22:136-140
245. Yoshike N, Matsumura Y, Iwaya M, Sugiyama M, Yamaguchi M, J. Epidemiol. 1996;6:S189-S200
246. Marr P, Middle States Geographer 2007;40:22-31
247. Seo J, Journal of Purdue Undergraduate Research 2011;1:38-45
248. Singewald J, Miller B, The Mineral Deposits of South America, 1st ed. McGraw-Hill Book Co., NY 1919
249. Hammame M, Report 2002:1 Environmental Systems Analysis, Chalmers Univ. of Tech.,Goteborg,Sweden
250. Armijo R, Gonzalez A, Orellana A, Coulson A, Sayre J, Detels R, Int. J. Epidemiol. 1981;10(1):57-62
251. Witkop C, Barros L, Hamilton P, Public Health Rep. 1962;77(11):928-940
252. Ward M, deKok T, Levallois P, Brender J, Gulis G, Nolan B VanDerslice J, Environ. Health Perspect. 2005;113:1607-1614
253. Ward M, Rev. Environ. Health 2009;24(4)357-363
254. Chilvers C, Inskip H, Caygill C, Bartholomew B, Fraser P, Hill M, Int. J. Epidemiol. 1984;13(3):324-331
255. Mahvi A, Nouri J, Babaei A, Nabizadeh R, Int. J. Environ. Sci. Tech. 2005;2(1):41-47
256. Water Quality Fact Sheet: Nitrate British Geological Survey 2004;1-6
257. Tirado R, Greenpeace Research Laboratories Technical Note 10/2007
258. Spalding R, Exner M, J. Environ. Qual. 1993;22(3)392-402
259. Helsel D, Nitrtae in the Nation's Waters: A Summary of Recent Studies, U.S. Geological Survey
260. Korom S, Water Resour. Res. 1992;28(6):1657-1668
261. Mahler R, Impacts and Management of Soil Acidity under Direct Seed Systems-Status and Effects on Crop Production. Presented at: Direct Seed Conference; Jan. 16, 2002: Spokane,WA.
262. Addiscott T, RSC 1996 ISBN 978-1-84755-008-8 DOI:10.1039/9781847550088-00001
263. Olesen J, Askegaard M, Nitrate Leaching from Arable Crop Rotation in Organic Farming, Dept. of Agroecology, Danish Institiue of Agricultural Science, Tjele,Denmark, http://orgprints.org/3857
264. Mitchell T, Harding A, Environmental Health Oct. 1996
265. Hinkle S, Quality of Shallow Ground Water in Alluvial Aquifers of the Willamette Basin, Oregon, 1993-95, U.S. Geological Survey Water-Resources Investigations Report 97-4082-B
266. Kite-Powell A, Harding A, JAWRA 2006;42(4):975-987
267. Tank G, Esselbaugh N, Warnick K, Storvick C, Cooperative Nutritional Status Studies In the Western Region III. Variation in Dental Caries Experience Among Children in Five Western States, Agricultural Experiment Station, Oregon State College, Corvallis, Tech. Bull. 45
268. Frans L, Estimating the Probability of Elevated Nitrate Concentrations in Ground Water in Washington State, USGS Scientific Investigations Report 2008-5025
269. Hard M, Esselbaugh N, Jacobson F, J. Am. Diet. Assoc. 1965;47(4):274-281
270. Hadjimarkos D, Fd. Cosmet. Toxicol. 1973;11:1083-1095
271. Hadjimarkos D, Shearer T, Am. J. Clin. Nutr. 1971;24:1210-1211
272. Hadjimarkos D, Arch. Environ. Health 1965;10:893-899
273. Hadjimarkos D, Storvick C, Remmert L, J. Pediatr. 1952;40(4):451-455
274. Hadjimarkos D, Bordens Rev. Nutr. Res. 1996;27(3):1-14
275. Selenium, Environmental Health Criteria Series, WHO, Geneva, 1987
276. Hojo Y, Sci. Total Environ. 1987;65:85-94
277. Freedman J., Frei B, Welch G, Loscalzo J, J. Clin. Invest. 1995;96:394-400
278. Khotimichenko S, Vopr. Pitan. 1997;4:16-18 PMID: 9412072
279. Suzuki K, Ogra Y, Food Addit. Contam. 2002;19(10):974-983
280. Eskandari S, Loo D, Dai G, Levy O, Wright E, Carrasco N, J. Biol. Chem. 1997;272(43):27230-27238

281. Hofbauer L, Spitzweg C, Magerstadt R, Heufelder A, Postgrad Med. J. 1997;73(856):103-104
282. Olivieri O, Girelli D, Stanzial A, Rossi L, Bassi A, Corrocher R, Biol. Trace Elem. Res. 1996;51(1):31-41
283. Contempre B, Duale N, Dumont J, Ngo B, Diplock A, Vanderpas J, Clin. Endocrinol.(Oxf). 1992;36(6):579-583
284. Olivieri O, Girelli D, Azzini M, Stanzial A, Russo C, Ferroni M, Corrocher R, Clin. Sci. (Lond). 1995;89(6):637-642
285. Wolff J, Maury J, Endocrinology 1966;79:795-800
286. Williams C, Thorton I, Plant and Soil 1972;36:395-406
287. Ludwig T, Healy W, Losee C, Nature 1960;186:695-696
288. Anderson R. Caries Res. 1969;3:75-87
289. Losee F, Bibby B, NY State Dent. J. 1970;36(1):15-19
290. Losee F, Ludwig T, JDR 1970;49(6):1229-1235
291. Corcuera G, Bastidas M, Dubourdieu M, J. Gen. Microbiol. 1993;139:1869-1875
292. Mendel R, Molybdenum: biological activity and metabolism. Presented at Dalton Discussion N0. 8, 7-9th Sept. 2005, Univ. of Nottingham, UK
293. Kalman L, Gunsalus R, J. Bacteriol. 1990;172(12):7049-7056
294. Lester R, DeMoss J, J. Bacteriol. 1971;105(3):1006-1014
295. Dwyer J, Krall E, Coleman K, J. Am. Diet. Assoc. 1987;87(11):1509-1512
296. Smiciklas-Wright H, Dietary Intake: Data Collection and Processing www.nutrientdataconf.org/PastConf/NDBC19/5-2_Smicklas-Wright.pdf
297. Packer P, Leach S, Duncan S, Thompson M, Hill M, Carcinogenesis 1989;10(11):1989-1996
298. Forman D, Cancer Surveys 1989;8(2):443-448
299. Mensinga T, Speijers G, Meulenbelt J, Toxicol. Rev. 2003;22(1):41-51
300. Eichholzer M, Gutwiller F, Nutr. Rev. 1998;56(4 Pt 1):95-105
301. Hord N, Tang Y, Bryan N, Am. J. Clin. Nutr. 2009;90:1-10