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NITRATES AND DRINKING WATER

SUMMARY

Nitrate levels in water sources are typically 0-10 mg l^{-1} in areas where there is no intensive agriculture. The experience of the water supply industry indicates that certain agricultural activities may lead to nitrate leaching resulting in concentrations approaching 50 mg l^{-1} and in certain instances exceeding 50 mg NO_3^- l^{-1} average over a season.

Nitrogen is an essential plant nutrient, mainly absorbed in the form of nitrate or ammonium ions and is used in protein synthesis. Plant proteins are used by animals and man as a dietary source of amino acids. The nitrogen absorbed by the plants from the soil must be replaced or agricultural productivity will not be maintained in the long term ; this is done by use of animal manure or inorganic fertilizers.

There are large quantities of nitrogen in soil. A proportion of this can be mineralised to nitrate each year; a process enhanced by tillage. Nitrate leaching increases as land use progresses from forest through grassland, to arable land, and to horticulture. The main source of nitrate leaching results from release of nitrate from soil organic matter in bare land especially during the winter. The next source of nitrate leaching to water systems occurs when the use of nitrogen fertilizer or organic manure exceeds the soil and crop capacity to utilise the nitrogen. Reduction of current fertilizer nitrate application levels would not markedly reduce nitrate leaching.

Denitrification by bacterial or geochemical mechanisms is an important natural process. Other ways of reducing the nitrate content of water are improvement of agricultural practice (prevention) or removal from water supplies (cure).

Nitrate is a normal constituent of human food, vegetables being the principle source. Estimates of dietary nitrate intake show it to lie between 30-300 mg NO_3^-/day depending upon dietary habits. The nitrate intake of vegetarians is considerably higher than that of non-vegetarians. When nitrate concentrations in potable water reach 50 mg NO_3 l^{-1} the water nitrate contributes about 55% to the daily total nitrate pool. In bottle

fed infants the water used in the preparation of the feed is the main source of nitrate. Milk itself contains less than 5 mg NO_3^- l^{-1}.

Nitrate synthesis occurs in animals and man. Nitrate balance is difficult to measure in man; it has been estimated that synthesis is about 50 mg NO_3^-/day. The rate of synthesis can be increased substantially during gastrointestinal infection.

Absorbed nitrate is excreted mostly unchanged in the urine, but some is reduced by bacteria to nitrite. All the health concerns regarding nitrate relate to this potential reduction to nitrite. The extent of nitrite production under various circumstances is not known.

Nitrite is a reactive molecule and can participate in numerous reactions with food components in the gastrointestinal tract. It is also taken up in the blood where it reacts with haemoglobin to form methaemoglobin.

There is no evidence from animal experiments that nitrate or nitrite cause cancer. Nitrate is not mutagenic. Recent epidemiological research provides no evidence that nitrate induces cancer in man.

In vitro nitrosation of dietary compounds occurs in human gastric juice yielding, among other possible reaction products, N-nitrosocompounds. Many such compounds are carcinogenic to animals. Man excretes some nitrosocompounds in urine, e.g. nitrosoproline ; this excretion is increased by simultaneous ingestion of high doses of nitrate and proline. At present it is not clear whether this excretion is influenced by variations in normal dietary nitrate intake. Although N-nitrosocompounds can be detected in several body fluids evidence suggests that injestion of nitrate does not significantly increase their concentration. Increased nitrite and nitrosocompounds levels have been detected in man suffering from a deficiency in gastric acid production.

In experiments on rodents, nitrate and nitrite reduce growth, litter size and increase relative kidney weight. Nitrite increases methaemoglobin levels and brings about histopathological changes in heart, lung, liver and kidney. The highest no-effect levels are 500 mg/kgbw for nitrate and 50 mg/kgbw for nitrite.

A principle health concern is the development of methaemoglobinaemia in infants receiving high nitrate intakes.

The normal methaemoglobin level in the blood is 0.5-2%. Use of water containing 50-100 mg NO_3^- l^{-1} for infant feed preparation results in methaemoglobin levels within the normal physiological range although possibly at the high end of the normal range.

When the methaemoglobin concentration in blood exceeds 10% the skin takes on a blue tinge, a condition termed methaemoglobinaemia. Nitrate does not produce methaemoglobinaemia; it has to be reduced to nitrite to induce the condition.

Acute infantile methaemoglobinaemia can be a rare complication in gastroenteritis irrespective of nitrate intake.

Acute infantile methaemoglobinaemia resulting from bottle feeding has been associated only with the use of high nitrate well-water and was termed well-water methaemoglobinaemia. The condition is rare and has decreased in incidence in Western Europe so that in the last 20 years it has become virtually non-existent. Most cases occurred when the well-water nitrate levels exceeded 100 mg l^{-1} ; in those cases where it was associated with lower concentrations the bacteriological status of the water was poor and/or the infants were suffering from gastroenteritis.

There is no evidence that infantile methaemoglobinaemia is caused by bacteriologically sound water supplies containing nitrate concentrations up to 100 mg l^{-1}. 98% of the European population is supplied by piped mains water treated to remove bacteriological contamination. The remaining 2% is supplied with well-water of variable quality.

The recent reductions in the acceptable upper limit for nitrate in drinking water from about 100 mg l^{-1} to about 50 mg l^{-1}, and the establishment of a guide level of 25 mg l^{-1} are not justified on two grounds. Firstly the clinical data show that infant methaemoglobin levels associated with nitrate concentrations in the range 50-100 mg l^{-1} fall within the normal physiological range (0.5-2.0%). Secondly in the few cases where the use of such water causes infantile methaemoglobinaemia the condition was

associated with either gastroenteritis or the use of water of poor hygienic quality, thereby casting doubts on nitrate as a causative factor in such cases.

1. INTRODUCTION

Nitrogen is an element essential for all known plant and animal life. Nitrate, generated from the soil organic matter, is naturally occurring in soil and water, and is required for primary production of biomass. Nitrate is not bound to soil and therefore moves through soil with the soil water. Plants require nitrogen for the build-up of chlorophyll, amino acids and other essential components. Yields of crops and the productivity of livestock have increased considerably in most of the world over the last 30 years. New agricultural technology, including the use of nitrogen containing fertilizers have made this progress possible. Agricultural productivity cannot be maintained without nitrogen, be it from farm yard manure, biological fixation or inorganic fertilizers.

Water not taken up by plants or lost by evaporation percolates through the soil into lower water bearing rocks, or runs to rivers. Both ground and river water enter public piped water supplies which is therefore affected by agricultural practice.

Piped water supply, as opposed to untreated domestic well-water, has been the principle form of water supply in Western Europe for the last 30 years and about 98% of the population now receive piped-water. Local legislation has controlled the quality of these supplies since the mid 19th century. More recently controls have been based on an EC Directive (EEC, 1980). Of the 62 parameters, nitrate is listed within a group of non-toxic substances the concentration of which may exceed that stated in the Directive at the discretion of individual Member States, provided there is no danger to public health. The Directive specifies a "maximum admissible concentration" for nitrate of 50 mg NO_3^- l^{-1}, in line with the "recommended" concentration specified by WHO (1970); it also defined a target guide level of 25 mg l^{-1} without defining the criteria by which this level was agreed.

Until 1984 the WHO standard for drinking water, whilst recommending a concentration below 50 mg NO_3^- l^{-1}, classified concentrations between 50 and 100 mg l^{-1} as conditionally acceptable. Concentrations in excess of 100 mg l^{-1} were not recommended. Subsequently WHO (1984) reduced the

recommended standard to 10 mg NO_3-N l^{-1} (45 mg NO_3 l^{-1}) and dispensed with the earlier defined acceptable range largely on the basis of an assumed risk of occurrence of infantile methaemoglobinaemia.

Current views on nitrate toxicity are not settled and ECETOC considered that a critical and comprehensive literature review might serve to clarify the situation and identify inconsistencies and possible mistakes in the earlier work and indicate areas where further research is desirable.

This report reviews the significance of nitrate in Western European water supplies, the role of agriculture, how nitrate reaches the water supply, nitrate intake by the human population, and its possible effect upon the man have been examined.

The terms of reference of the Task Force were to :

- establish the main sources of nitrate and quantify the nitrate content of ground and drinking water in Western Europe.

- define the main sources of nitrate intake by the Western European Consumer and the total daily nitrate intake.

- review the evidence that nitrate intake effects the health status of either individuals or populations.

- review critical evidence in relation to WHO recommendations on nitrate in drinking water, and if appropriate explain why conclusions differ.

Units

The units used to express nitrate concentration are a source of confusion. Concentration has been expressed in moles, mg NO_3^- l^{-1} or mg NO_3-N l^{-1}. WHO (1985) adopted the latter. We have chosen the conventional mg NO_3^- l^{-1} as it is employed in most of the original papers and reflects the established usage in European Water Quality

Regulations. The disadvantage is that concentrations of NO_3 and NO_2 are not immediately comparable on a molar basis.

For conversion from one system to another the following factors can be used:

1 mg NO_3^- l^{-1}	=	0.226 mg NO_3^- - N l^{-1}	
1 mg NO_3-N l^{-1}	=	4.429 mg NO_3^- l^{-1}	
1 m mole l^{-1}	=	62 mg NO_3^- l^{-1}	= 46 mg NO_2^- l^{-1}
	=	14 mg NO_3 - N l^{-1}	= 14 mg NO_2^- N l^{-1}

Glossary

Some of the agricultural, medical and chemical terms used in this document are not in every day use and are defined in the glossary (Appendix 1) to assist readers.

Literature

Much of the medical evidence cited in this and other documents (eg WHO 1970, 1985) has not been peer reviewed. Many of the reports are based on evidence as seen by the clinical investigators conducting the work at the time which with the benefit of hindsight, is often lacking in important material evidence.

The literature up to the end of 1986 should be well covered. Later papers (up to the autumn of 1987) are also included but the coverage may not be complete.

2. NITRATE CONTENT OF EUROPEAN WATER.

2.1 Background

Although this is a well investigated topic the information published in the scientific literature is inadequate and incomplete. Most information has been generated by the public water undertakings and supplied routinely to Government Departments. Summarized data for a number of European countries is given in Appendix 2.

A recent UK Government report (UK-DOE, 1986) has examined nitrate concentrations in public water supplies, current trends and future predictions. All UK public water supplies comply with a rolling 3-monthly mean nitrate concentration not exceeding 80 mg NO_3^- l^{-1}, with no individual sample exceeding 100 mg NO_3^- l^{-1}. Rivers show a seasonal pattern of nitrate levels; these are highest in the winter when drainage from the land occurs during autumn and winter rains. Fig 1 shows a long term rise and the annual variation in nitrate levels for a UK river showing high concentrations of nitrate. It will be seen that peak winter levels can exceed 100 mg NO_3^- l^{-1} and during such periods it is necessary to restrict supply to waterworks which draw water directly from the river and to provide alternative supplies.

In underground waters elevated nitrate levels are found where arable land overlies the water-bearing rock (aquifer) and there is no intervening clay barrier. High nitrate water percolates downwards and, depending on the thickness of the overlying rock takes between 5 and 40 years to reach the water table.

The extent of nitrate accumulation depends on climatic and geological conditions. Mathematical models predict that for the most severely affected area in UK levels may eventually reach about 150 mg NO_3^- l^{-1} ; this assumes a continuation of present agricultural practice (Foster et al, 1986). Over the last 50 years the level of nitrate in rivers and in water pumped from deep wells has risen greatly. The extent of the rise is highly variable and depends on a range of factors. There is some evidence that this trend is changing; data from monitoring at 149 stations on rivers in England, Wales and Scotland shows no significant

change in the average nitrate content of river water from 1977 to 1984 although regional variations exist (UK, DOE, 1986). This apparent plateau has occurred even though fertilizer use (nitrogen/hectare) has increased considerably especially in the arable farming areas. Evidence showing a similar trend is available from some rivers in the Federal Republic of Germany (Appendix 2).

Practical and financial constraints limit the actions that may be taken by authorities in providing water to the EEC standard. Possible actions are :

1. replacement of high nitrate sources, by diverting water from low nitrate supplies by means of trunk mains.

2. controlled blending of low and high nitrate waters; this has been practised by several water authorities, however, this can only be a relatively short term measure if the general levels of nitrate in water continue to rise.

3. maximise natural denitrification by long storage in large reservoirs.

4. provide additional water treatment processes, e.g. ion exchange processes (Greene, 1980), or microbiological denitrification (Wilkinson et al, 1982). The long term health implications of such processes are unknown, so they need to be implemented with care.

5. provision of bottled water to populations identified at risk, particularly to infants under 6 months of age.

Competent authorities have to assess the effectiveness and costs of the various actions they might take to limit nitrate intake. Apparent risks (cf sections 6, 7 and 8) and the sources of contamination have to be taken into account. Closure of high nitrate sources and their replacement by low nitrate sources implies provision of new trunk water mains and the availability of low nitrate supplies. Blending also calls for alternative sources and facilities which operate seasonally.

Biological denitrification processes occur naturally within the muds when water is stored in reservoirs (Vollenweider, 1968). Both ion exchange and denitrification processes are feasible, but the costs substantial, the former is suitable for both ground and surface waters supplies and is preferred for small installations for operational reasons; the process is complicated by the need to dispose of spent regenerant brine and to maintain the final water quality. Microbiological denitrification is an alternative for surface derived waters, particularly for larger plants. Start-up is slower than for ion exchange and further physico-chemical treatment of the denitrified water is necessary. Operation and maintenance are more onerous, requiring the strict monitoring of residual methanol levels in the water. Operating experience has indicated a need for careful maintenance; this restricts such processes to manned water treatment works. Operational experience has demonstrated problems associated with intermittent high nitrite levels in the water to be treated and poor performance of monitoring equipment.

2.2 Factors influencing water Nitrate Levels

As will be described in section 7, the main public health concern arises from water derived from private wells, which supply 2% of the EEC population (based on a European Community of 10 States, excluding Spain and Portugal, for which information is not easily available).

The alternative to removal of nitrate during water treatment is reduction of the input of nitrate into the environment. This is linked inevitably to agricultural productivity and land use. A reduction of fertilizer usage would probably have minor effects on nitrate levels for the following reasons :

a. The organic nitrogen reservoir in the soil is large, and in highly productive soil systems is bound to organic material that would take many years to diminish.

b. An arable agriculture even with reduced fertilizer usage and crop yields could still produce nitrate levels in excess of 80 mg NO_3^- l^{-1}.

c. The nitrate from present drainage has not yet reached the water table. In some areas this may not happen for forty years.

A worst case situation is exemplified by the Eastern Counties of the UK, where the extent of annual drainage is typically less than 250 mm and farming is predominantly arable in character. In this situation to achieve a major effect on nitrate leaching it would be necessary to change to permanent grassland or forestry. It can be calculated that the loss of 11.3 kg N/hectare as NO_3^- from agricultural land per 100 mm of drainage water results in an average concentration of 50 mg NO_3^- l^{-1} in drainage water. Losses from arable land are in the range 10-80 kg NO_3-N per hectare at 1 m depth whilst grassland and forestry areas are likely to lose less than 10 kg NO_3-N per hectare (Cooke, 1974; Owens and Jürgens-Gschwind, 1986). The areas which would need to be converted would depend upon the nitrate levels to be achieved. If 50 mg NO_3^- l^{-1} of water is the target then several thousands of square kilometers of prime arable land in the UK alone would need to be changed. If 80 mg NO_3^- l^{-1} were the target then a smaller but still significant area of land would require conversion. The exact areas needing conversion are the subject of current investigation. Simply reducing nitrate fertilizer application rates would not markedly reduce nitrate leaching; this topic is further explained in section 3. In the long term, water undertakings utilizing ground waters in agricultural areas may be forced to remove nitrate by specific treatment processes.

2.3 Summary

- 98% of the Western European population is supplied by mains piped water; the remaining 2% is supplied by well water.
- the concentration of nitrate has risen in river and ground waters in most European countries over the past years.
- although nitrate concentration in water for some regions have tended to rise there is some evidence, from both Germany and UK, that the rate of increase is reducing.
- nitrate can be removed from drinking water at a cost but the various techniques may produce their own problems that have not yet been evaluated.

- organic nitrogen is the major source of nitrate; reduction of fertilizer application rates will not reduce water nitrate levels substantially in the short term.

3. AGRICULTURAL PRACTICE AND WATER NITRATE LEVELS

3.1 The soil nitrogen cycle

In the conventional nitrogen cycle, nitrate N is taken up by plants, incorporated into organic substances, and later transferred to animals and man. In an undisturbed environment it is returned to soil as animal excretion products or dead animal and plant material. This natural cycle is broken when man removes the nutrients from their original site with the harvest material (figure 2) (Owen and Jürgens-Gschwind, 1986). If plant nutrients are not replaced with either organic or mineral fertilizer the soil will loose its nutrient reserves and soil fertility decline. One of the main tasks of agriculture is thus to re-connect the interrupted circulation of nutrients by fertilization. Mineral nitrogen fertilizers provide plants with the same nutrient form as is produced by the decay of organic substances in the soil.

Largely due to more effective soil cultivation techniques the top soil of central European farms is three times as deep compared to 100 years ago. Increased fertilization, both organic and mineral, has enriched the deeper top soil with minerals and humus. Thus according to Huppert and Jürgens-Gschwind (1965) and Mineev (1979) :

More Nitrogen → More Silage, straw stubble and root residues → More Cattle and manure → More humus → Improved soil structure and higher soil fertility, higher yields.

The organic residues and humus content, after decay and mineralization, form the main source of mobile nitrate which has become a principle topic in discussion on environment and drinking-water quality (Kuntze, 1983; Welte and Timmerman, 1982). All soils contain large amounts of organically bound nitrogen, arable top soils contain 3000-8000 kg N/ha whilst grassland soils contain up to 15,000 kg N/ha; 90-95% of the

nitrogen is bound in organic matter, principally humus (Amberger, 1983a; Kuntze, 1983; Kolenbrander, 1972; Low, 1976). Depending on site and climate, 1-3% per year of the organically bound nitrogen is mineralized to ammonia, which is then nitrified to nitrate. The extent of mineralisation depends on many factors (soil, weather, previous crops) and is difficult to predict accurately. It is the effect of agricultural activities, specifically soil tillage, that releases this nitrogen store as nitrate (Cooke, 1984; Vetter, 1985; Welte and Timmerman, 1982). The nitrate may be taken up by plants, biologically fixed as microbial mass, denitrified by microorganisms, or leached from the soil. Mineralisation is a slow process, whilst nitrification is rapid.

It has been concluded from long-term trials in which wheat or rye were grown on soil fertilized normally for years that on unfertilized soil mineralization of the organic matter exceeds the amount of nitrogen bound by new organic material in the first ten to twenty years. The nitrogen released is used by the plants or is leached and/or denitrified. Eventually N-mineralization and N-immobilization reach an equilibrium (Hera et al, 1981; Jenkinson, 1982). All forms of mechanised soil cultivation increase the breakdown of humus, leading to more available nitrogen (Kanwar, 1985; Knittel et al, 1985; Morel, 1976). Mineral fertilization initially decreases the amount of organic matter due to stimulation of microbial activity and later increases organic matter by increasing the harvest residues.

3.2 Behaviour of Nitrate in Soil

Nitrate ions that are not taken up by plants or micro-organisms remain in and move with the soil water. Downward movement predominates in any vegetation-free period. Under plant cover the upward movement predominates as a result of root uptake and the evaporation of water and the temperature gradient between soil and air. Generally the more water that drains into the deeper soil layers from the topsoil, the more nitrate is lost due to leaching (Amberger 1974; Kjellerup and Dam Kofoed, 1983).

3.2.1 Nitrate leaching as influenced by Crop Cover

Nitrate is leached from all soils, including those under natural vegetation and those lying fallow that have never received any fertilizer (Czeratzki, 1973 ; Furrer, 1986; Otto, 1978). It is the extent that differs (Jürgens-Gschwind and Jung, 1977). In general bare soils release greater amounts of leachable nitrate than cropped soils because of the lack of competitive N uptake of plant roots (Atkins, 1976; Cooke, 1984; Remy, 1984; Triboi and Gachon, 1985), (figure 3).

Intensively growing crops take up about 1-5 kg N per hectare per day and thereby largely prevent nitrate from being leached (Amberger, 1983); if more nitrogen (either fertilizer or organic manure) is applied than the crop can use leaching may occur (Kolenbrander, 1981; Schweiger, 1985). Presence of a crop and the type of plant thus have far more influence on the amount of nitrate leached than other agricultural practices, including nitrate application (Stauffer and Furrer, 1981; Furrer, 1986). The leaching of nitrogen increases progressively from woodland through grassland to arable land and is highest under intensive horticultural cultivation (Bramm, 1979; Dressel and Jung, 1984; Otto, 1981; Sontheimer and Rohmann, 1984; Vetter, 1985). Arable land loses 1/3rd and grassland 1/10th the amount of nitrogen lost by fallow land (Czeratzki, 1973; Hoffer and Jäggli, 1975; Kolenbrander, 1981; Low and Armitage, 1970). The type of crop and rotation considerably affect the leaching process on arable land (Amberger, 1983; Atkins, 1976; Cooke, 1984; Jung and Jürgens-Gschwind, 1974; Ohlendorf, 1976; Welte and Timmermann, 1982). Vigorous growth of a long lasting plant cover (System Evergreen) is the best tool to prevent or reduce leaching of nitrate into deeper soil zones (Jürgens-Gschwind and Jung, 1977; Pfaff, 1963; Remy, 1984; Stauffer and Furrer, 1981; Triboi and Gachon, 1985). In contrast, after the termination of active growth nitrate uptake ceases and subsequent leaching becomes possible. This is true particularly after the harvest.

Good agricultural management therefore tries to counteract this by appropriate measures. For instance planting winter-cereal crops in

the early autumn allows growth of the seed in the early fall and consequently uptake of nitrate that otherwise could leach to water supplies. Similarly where a following crop would be sown in the spring (e.g. sugar beet, potatoes, spring cereal, maize), sowing an intermediate cover crop (at some cost) may reduce nitrogen leaching by 50-75% (Berendonk, 1985). Special attention needs to be drawn to the legumes which may increase nitrate leaching since these plants fix atmospheric nitrogen with the help of symbiotic bacteria in their root nodules, some of which is released to the soil as nitrate after the plants have died (Furrer and Stauffer, 1985; Furrer, 1986). It seems reasonable to forecast that extended planting of potential new plant varieties incorporating the nitrogen fixing mechanism will likewise result in increased nitrate leakage.

Construction areas, railway tracks and non-planted sports grounds behave in the same way as fallow land. It was found that drainage and well-water from built-up areas may contain more nitrate than that from agricultural land (Bolzer, 1981; Henin, 1981; Kemmerling, 1983; Vetter and Steffens, 1983).

3.2.2 Nitrate Leaching as influenced by Rainfall and Irrigation.

In Europe autumn/winter rainfall normally exceeds the evaporation rate and any nitrate present in the top soil is at risk of being leached (Jung et al, 1969; Pfaff, 1963). As a general rule the greater the winter rainfall the greater will be the amount of nitrate leached from the soil (Jung, 1972; Paltineanu et al, 1973). The average concentration of nitrate in the leachate is likely to decline, both as the winter progresses and rainfall volume increases. The leaching effect can be observed even when no nitrogen fertilizer is applied (Jung et al, 1969; Jung and Jürgens-Gschwind, 1974; Kjellerup and Dam Kofoed, 1983).

Carefully targetted irrigation to meet the crop needs is likely to maximise crop nitrate uptake and minimise leaching losses (Jürgens-Gschwind and Jung, 1977; Pfaff, 1958, 1963). Application of sufficient nitrogen under these conditions leads to greater uptake of nitrate by the crop and reduced nitrate leaching (Bramm, 1981;

Buchner and Sturm, 1985; Jung and Jürgens-Gschwind, 1974; Webster et al, 1984). On the other hand excessive irrigation is not economic and may increase leaching losses (Atkins, 1976; Paltineanu et al, 1973) as may rainfall following irrigation. Experiments on arable and horticultural crops showed a decrease of the nitrate concentration of 5-50% in the leachate from irrigated fields (Pfaff, 1958; Czeratzki et al, 1976). On grassland on a sandy soil at the fertilizer usage rate of 400 kg N/ha/y the decrease amounted to nearly 20% (Steenvoorder, 1987). Because irrigation is likely to increase the amount of water draining from the soil in the drainage season (Bailey, 1987), it is possible that irrigation may increase slightly the total nitrate loss, though the nitrate concentrations in the drainage water may be slightly lower.

3.2.3 Nitrate Leaching as influenced by Temperature

Temperature affects leaching mainly through changes in the amount of nitrate present in the top soil. This depends on the balance between nitrogen mineralization and immobilization in the soil and crop nitrate uptake. All of these processes increase as the soil temperature rises above 5°C. Growth of temperate plants is likely to be retarded at temperatures above 28°C and in hot summers nitrate accumulation may take place, thereby increasing the risks of losses in the following winter.

Temperatures below 5°C slow down or inhibit these processes, whilst prolonged freezing conditions stop movement of water through the soil so halting leaching. The subsequent risk of nitrate leaching loss will depend on the amount and distribution of spring rainfall (Buchner and Sturm, 1985; Jung et al, 1969; Otto, 1978).

3.2.4 Nitrate Leaching as influenced by Type of Soil

The main soil characteristics that affect nitrate leaching are soil depth, geological source (parent material), texture and structure. Shallow soils are usually freely drained and nitrate when present is easily leached to below the reach of plant roots. Organic soils, because of their high nitrogen content, typically mineralise more

nitrogen than mineral soils and hence there is greater risk of nitrate loss from organic than from mineral soils (Allison, 1966; Amberger and Schweiger, 1978; Hera et al, 1981; Jung and Jürgens-Gschwind, 1974; Kolenbrander, 1981; Mündel, 1987; Royal Society Study Group, 1983; Spallacci and Boschi, 1980).

Texture and structure affect the amount and movement of water in the soil. Sandy/light textured soils on the whole are less retentive of water than clayey/heavy textured soils. Sandy soils tend to be poorly structured and have a fairly uniform porosity. This means that water moves easily through the soil and nitrate can be leached out completely by a small volume of rainfall. By contrast the finer particles of clayey soils aggregate together to form lumps that give the soil its structure. This results in a range of pore sizes; the small pores remain water filled, even in the air dry soil, while surplus water can move freely through large cracks and spaces. The soil is therefore more retentive of both water and nitrate and a large amount of rainfall is needed to leach out nitrate completely. In practice, this means that with sandy soils the nitrate concentrations are highest in water draining initially and then decline rapidly to low levels. With clay soils the concentration of nitrate in the initial drainage is also higher than in later drainage but lower compared to sandy soils and the rate of decline in concentration is slower.

3.2.5 Nitrate Concentrations Leaching from Soils

It is important to recognise that although total amounts of nitrogen lost may be low, expressed for example on an annual basis, peak leaching losses may be substantial. Where soils overlie non-porous subsoil and water is drained into streams and rivers that are used as a water supply it is the concentration of nitrate leaching from the soil that is important. Thus when drainage starts in the autumn concentrations often exceed 50 mg l^{-1} and may approach 200 mg l^{-1} even though the mean annual concentration does not exceed 50 mg l^{-1}. In contrast when soils overlay a porous subsoil that forms an aquifer the effect of the peak autumn concentrations is lost as the mean concentration of nitrate in the aquifer is measured.

3.2.6 Nitrate Leaching as Influenced by Ground water Level and Drainage

Factors which increase the residence time of water in the soil reduce losses of nitrate by leaching. This is due to increased uptake of nitrate by plants and to greater losses by denitrification. For these reasons soils with a low groundwater table, a large water holding capacity, or poor natural drainage all tend to show lower nitrate losses (Kolenbrander, 1980; Rijtema, 1980). In contrast, the installation of artificial drainage carries excess water away more quickly and leads to increased nitrate leaching losses (Colbourn, 1985; Garwood, 1984).

In addition, in low-lying areas (e.g. in the Netherlands) changes in the water table counteract the leaching of nitrate. This is explained by greater capillary rise and better conditions for denitrification in the upper part of the soil (Steenvoorden, 1987a).

3.2.7 Nitrates Leaching as Influenced by Amount, Timing and Form of Manuring and Fertilizer Use.

Provided fertilizer application rates do not exceed the capacity of the soil-plant system to utilise the nitrate then the effect of mineral fertilization on leaching losses is minimal. If fertilizer is applied in excess then leaching will occur.

Improvements in crop production aided by modern plant varieties, better soil management and optimum cultivation techniques remove large amounts of nitrate from the soil (Bommer, 1982; Buchner and Sturm, 1985; Zerulla, 1986). For example long-term trials at Rothamsted, England, showed that wheat (grain and straw) could only use 32% of the N fertilizer supply during the period 1852-67 yet by 1979-80, this had increased to 86% (Jenkinson, 1982). In general the distribution of nitrogen administered is

taken up by the crop plants	50-75 %,
bound in the organic matter of the soil	5-25 %,
lost as gas to the air after denitrification has taken place	2-20 %,
leached into the subsoil	2-10 %.

(Colbourn, 1985; Kjellerup and Dam Kofoed, 1983; Remy, 1984; Webster et al, 1986; Welte and Timmermann, 1982).

The optimum timing of the N fertilization is important to obtain the best possible utilization by the crop and thus minimize nitrate concentration of water sources. It can be advantageous to divide the fertilizer requirements into several applications through the growing season (Buchner and Sturm, 1985; Cooke, 1984). It is usually no longer justifiable to apply nitrogen fertilizer in the autumn; this applies for both mineral fertilizer and organic manure (van Dijk and Sturm, 1983; Kjellerup and Dam Kofoed, 1983; Kolenbrander, 1981; Kuntze, 1986; Otto, 1981; Vetter and Steffens, 1986).

The form of nitrogen (nitrate, ammonium, urea) has relatively little effect on the leaching process, since urea and ammonium nitrogen are subject to rapid nitrification in the soil. However, a slightly higher level of leaching from nitrate as opposed to other forms has been confirmed (Atkins, 1976; Dressel and Jürgens-Gschwind, 1985; Jürgens-Geschwind and Jung, 1977; Lambrecht et al, 1979).

Organic manure is characterised by high bulk, low nutrient concentration, and high water content, all of which restrict the time and mode of use. It is usually applied to ploughed land in the autumn or winter as this avoids the risk of damage to crops. As the manure is not applied in accordance with the plant's needs the nitrogen is not utilized efficiently (Dam Kofoed, 1981; van Dijk and Sturm, 1983; Duthion, 1985; Steenvoorden, 1987a; Vetter and Steffens, 1986). As a consequence application time should be delayed to early spring. The amount of nitrogen available is difficult to calculate due to variation in composition, application rate and application time. Liquid organic manure, when applied in autumn, is only about 30% as effective as mineral fertilizer, its mineralization to nitrate causes severe leaching from open land during the succeeding wet winter (Vetter and Steffens, 1983). If the slurry is applied in the spring, coinciding with plant growth, it can be 70% as effective (van Dijk and Sturm, 1983). The effectiveness may be further increased by a more uniform distribution with modern machinery (Smith and Urwin, 1983; Vetter,

1985); direct soil injection of liquid organic manure can reduce leaching considerably (Steenvoorden, 1987a).

Nitrification inhibitors like 2-chloro-6-(trichloromethyl)-pyridine or dicyandiamide (DCD) retard the activity of soil microbes that oxidize ammonium to nitrite, e.g. Nitrosomonas sp. DCD decomposes in the soil to nitrate. The application of nitrification inhibitors together with liquid organic manure or liquid ammonia in the autumn prevents mineralization of the organic material in the soil for several weeks thus retarding N release. As a consequence nitrate availability is more gradual and can coincide with plant growth requirements in the spring. This may give reduced N losses by denitrification and leaching (Amberger, 1984; Timmermann and Söchtig, 1983). On a sandy soil leaching was reduced by about 27% within a 7 year period (Kuntze and Scheffer, 1987). In areas with existing high nitrate levels nitrification inhibitors cannot prevent the leaching risk. The advantages and problems with the use of nitrification inhibitors are currently an active field for agronomic research.

3.2.8 Nitrate Leaching as influenced by Animal Farming

During the last few decades the amount of manure produced by animal farming within the EEC has increased due to a higher animal population. Some farms carry no stock, whilst others are intensively stocked and the land area suitable for organic manure disposal is limited or non-existent. Thus animal manure, which was formerly considered and used as a valuable fertilizer, is considered as a waste product and an encumbrance. If there is not sufficient surrounding land the material may be dumped causing serious nitrate leaching. Generally the dumped organic manure is applied to the soil in autumn and winter with high risk of leaching losses.

On grassland, nitrogen fertilizer increases grass growth, most of the latter is consumed directly by grazing animals. The additional grass growth resulting from fertilizer application permits higher stocking densities. For cattle approximately 80% of the N consumed in feed is excreted and is potentially available for plant uptake.

The N in the animal manure is not efficiently used due to its high N concentrations and evaporation of NH_3. On intensive fertilized grassland (4 cattle/hectare fed supplementary concentrates) there can be an excess input of nitrogen and on sandy soils the leaching of nitrate can then approach levels observed under arable land (Cooke, 1984; Kolenbrander, 1981; Ryden et al, 1984; Steenvoorden, 1987a). However, on grassland continuously cut (i.e. without grazing animals) a long term study showed an optimum fertilizer rate of 420 kg N/hectare. Up to this level there was little risk of high nitrate accumulation in either the herbage or the soil (Prins, 1983; van Burg, 1986).

3.3 Nitrate Migration to Deeper Soil Zones

The movement of nitrate to deeper levels of the soil proceeds at 0.3-3 m/year (Landreau, 1983; Rohmann and Sontheimer, 1985; Young and Morgan-Jones, 1980). Agricultural practice has a significant effect upon this migration, e.g. the length of time soil is covered by growing plants, rooting intensity of the crop, growing conditions and the rotation system (Czeratzki, 1973; Remy, 1984; Furrer, 1986; Jürgens-Gschwind and Jung, 1977; Welte and Timmermann, 1982). Deforestation, ploughing-up of grassland, planting of legumes, and soil tillage increase leaching (Furrer and Stauffer, 1985; Kanwar, 1985; Low and Armitage, 1970; Stauffer and Furrer, 1981). It is estimated that in the Federal Republic of Germany about 1 million hectares of grassland have been transformed into arable land since 1950 leading to mineralisation of 5 mt N which is equivalent to three times the amount of inorganic fertilizer used in West Germany during 1980. These liberated reserves are believed to have contributed to the nitrate enrichment in several aquifers (Kuntze, 1986). In South-East England, nitrate enrichment in a chalk soil profile in the 12 years following the ploughing-up of grassland was mainly due to the mineralization of organic matter (Foster et al, 1982).

The fate of nitrate nitrogen passing through soil profile to the upper aquifer is still little known, except that it is very complex (CNGE/ENSCR, 1985; Lind and Pedersen, 1976; Obermann, 1985; Pedersen and Lind, 1976). In soil zones with a low oxygen content and relatively

high content of organic carbon the biological denitrifying process reduces and eliminates much of the percolating nitrate. The subsoil capacity for these reactions is limited and examples are known where it may have come close to exhaustion (Isermann, 1987; Rohmann and Sontheimer, 1985; Sontheimer and Rohmann, 1984).

Soil structure and the bedrock also play a part in the denitrification process (Tredoux and Kirchner, 1985). The length of time that water remains in the soil pores, particularly of chemically active layers, is important. Thus under anaerobic groundwater conditions, e.g. in homogeneous clay deposits, chemical denitrification may occur and reduce the NO_3^- content of the leachate to low values (Lind and Pedersen, 1976; Pedersen and Lind, 1976). This is being used by some water authorities to effect in situ denitrification.

3.4 Agricultural Practices that Minimize Soil Nitrate Leaching

Application of the following principles of good agricultural practice, as local circumstances allow, will reduce nitrate leaching to a practical minimum :

- Fallow periods should be avoided. The soil should be covered as long as possible (particularly during the winter months) by early sowing of winter cereals, intercropping, straw mulch, etc ("System Evergreen").
- Whilst doing this avoid increased sowing of legumes unless a subsequent crop can utilise efficiently the nitrogen released by mineralization of the nitrogen rich root residues.
- Grassland should not be ploughed.
- Soil tillage should be minimised and avoided in the autumn; direct drilling should be used where appropriate.
- Slopes should be cultivated in a transverse direction to minimise run-off.
- Organic manure should not be spread in autumn or winter. The amount applied should be spread evenly and not exceed the crop needs.
- Fertilizer and manure should be applied at times and in amounts appropriate to the nutrient requirements of the crop taking into

account the amount of available nitrogen already present in the soil.

- Use of nitrification inhibitors to delay mineralization may prove to be of value.

3.5 Summary

Soils contain large amounts of organically bound nitrogen, of which between 1 and 3% is converted to nitrate annually.

Nutrients including nitrogen are removed from the soil by the harvested crop. Replacement of nutrients by organic manure and fertilizer is necessary to maintain soil fertility.

Nitrate is not bound to the soil and will follow water movements. Nitrate can therefore leach when the soil receives more water than it can take up. This happens (in Europe) mainly in the late autumn, winter and early spring.

Grasslands and forests usually leach little nitrate because the mass of living plant roots take up most available mineral nitrogen, but tilled fertile fields give nitrate leakage. This is especially true for fallow land. Plants fixing nitrogen biologically (legumes) leave nitrogen rich root residues that can result in increased leakage.

Nitrate leakage from fields increases when manure or fertilizer is applied at times when the plants cannot make use of the nutrients, or in excess of plant needs. When properly timed and applied in amounts up to the levels recommended for optimal harvest, there is little or no increased field nitrate leakage. Reduction of nutrient use below this optimum will therefore not markedly reduce nitrate leakage.

Good agricultural practices (in which fertilizer management and maximising field plant coverage are important elements) will keep nitrate leakage associated with farming to the practicable minimum but actual levels achievable have yet to be defined.

To achieve levels of nitrate in water below the 50 mg l^{-1} EEC limit will almost certainly necessitate converting substantial areas of arable farmland into grassland with modest livestock numbers and/or establish forests. For surface waters changes would be apparent in a few years. For some aquifers changes should be apparent after 20 years (e.g. limestone), whilst for others (e.g. deep chalk) it may take 40+ years for levels to fall significantly.

4. NITRATE AND NITRITE INTAKE BY HUMAN BEINGS.

Both nitrate and nitrite are present in food and water consumed by human beings.

4.1 Nitrate and Nitrite Content of Water and Food.

4.1.1 Analytical procedures

Much of the historic information relating to the nitrate content of foods and amounts ingested has been obtained using analytical methods which are imprecise and insensitive and much of the data must be treated with caution. The review of analytical procedures (Appendix 3) indicates the performance of various methods.

4.1.2 Occurrence of nitrate and nitrite in foods

Vegetables and cured meat products are the main source of nitrate and nitrite in the diet but small amounts may be present in dairy and fish products. Table 1 shows the levels in various dairy and meat products whilst Table 2 levels in various vegetables as summarized by Corré and Briemer (1979). Water can also be a significant source of nitrate (see 4.1.3).

Dairy Products

Liquid milk does not generally contain more than 1-5 mg NO_3^- kg^{-1}. The level of nitrate in the diet of dairy cows appears to have only a very small effect on the nitrate concentration in the milk they produce (NAS, 1981).

Some cheeses may contain modest levels of nitrate and/or nitrite; nitrite may be added in some countries (Belgium, Denmark, Netherlands, Norway, Ireland, United Kingdom). Furthermore data from Denmark shows that nitrate and nitrite can be present in cheese made without nitrate or nitrite additions (Denmark SFI (1981), quoted by NAS, 1981).

Meat and Meat Products

Both nitrate and nitrite have long been used to cure meat e.g. bacon, ham and various types of sausage. The addition of nitrite is necessary to stop the growth of Clostridium botulinum which can cause botulism. There are regulations controlling the addition of sodium and potassium nitrates and nitrites to food products and these vary both between countries and between food products (BFMIRA, 1984). The data in Table 1 shows the mean range of concentrations present; within these means there is also a considerable range. For example von Collet (1983) gave the mean value for 23 samples of raw ham of 343 and 13.9 mg kg^{-1} for nitrate and nitrite respectively with an observed range from 20.3-1384 and 1.2-80 mg kg^{-1} respectively.

Fish

There are little published data on the nitrate and/or nitrite content of fish. Nitrate and/or nitrite are permitted additives to fish products in a number of countries (Finland, Federal Republic of Germany, Greece, Japan, Luxemburg and USA).

Vegetables

The literature on the nitrate content of vegetables has been authoritatively reviewed by Corre and Breimer (1979) who grouped fresh vegetables into 5 classes (Table 2). Subsequent data published do not conflict with those of Corre and Breimer.

Nitrite occurs in healthy living plants at low concentrations; typically 1-2 mg kg^{-1} may be found in fresh vegetables and 10 mg kg^{-1} is rarely exceeded (Corre and Breimer, 1979). Levels of 2-3 mg kg^{-1} may be found frequently in processed and stored vegetables.

Factors Affecting Nitrate Content of Vegetables

Processing. The processing e.g. washing, blanching and cooking of vegetables usually decreases the nitrate concentration of

vegetables. An increase can occur if the total water content is reduced by evaporation.

Nitrate is not uniformally distributed within the plant. In both cabbages and lettuces, for example, the high nitrate containing stems and outer leaves are often discarded when preparing food.

Growing conditions. Dry conditions favour nitrate accumulation. Crops grown in poor light or in glasshouses tend to have higher nitrate content.

Fertilizers. Nitrate is likely to accumulate in plant tissues wherever the amount of nitrate available in the soil substantially exceeds the plants requirement. Consequently a high nitrate content is most likely with high fertilizer application rates from either organic or inorganic sources. Nitrate concentrations are relatively low when fertilizer N application is optimised for economic crop production (Greenwood and Hunt, 1986).

Cereals and cereal products. Nitrate does not accumulate in the grains of cereals and is normally present in only low concentrations in flour. It has been observed that baking increases the nitrite content, compared with the original flour (Selenka and Brand Grimm, 1976), but there is very little published data on the content of baked goods. In 1975 White commented on this and when estimating the average nitrate intake of US citizens used values of 20 mg kg^{-1} for nitrate and 0.17 mg kg^{-1} for nitrite, based on earlier published American data. The NAS (1981) averaged available data and obtained 12 mg kg^{-1} for nitrate and 2.6 mg kg^{-1} for nitrite.

Beverages and Juices. As the principle beverages tea, coffee and beer, contain nitrates from the water from which they are made the levels will vary. Fruit juices contain about 2 mg NO_3^- l^{-1}. A survey in the UK (MAFF, 1987) quoted a mean of 30 mg NO_3^- l^{-1} and range of <10 - 100 mg NO_3^- l^{-1} for the nitrate content of beers.

Water. The nitrate content of water drawn from areas not influenced by agriculture can vary from 0-10 mg l^{-1} compared with 25 to over 100 mg l^{-1} from intensively farmed areas. Values over 2000 mg NO_3 l^{-1} have been found in severely polluted well-water contaminated by animal manure or sewage effluents.

Nitrite is only found in trace amounts (0-0.1 mg l^{-1}) in drinking water but might occur at higher levels in grossly polluted water.

4.1.3 Daily intake of nitrate and nitrite

Three approaches have been used to quantify the dietary intake of nitrate and nitrite.

1) the duplicated portion technique, where one portion is for consumption and the second one is for analysis (WHO, 1985a);
2) analysis of all components of the diet and using data about consumption of each component calculating the likely intake.
3) measurement of urinary nitrate load and calculation of total intake based on observations of the proportion of nitrate excreted in the urine (Bartholemew et al, 1979).

The first approach has the advantage that it takes full account of the changes brought about by food preparation and cooking but experience has shown it is difficult to monitor long enough or widely enough to obtain completely representative data. Some data obtained by this method is shown in Table 3. The values probably represent the extremes for average diets

The second approach is more empirical but it does allow estimates to be made for various diets. It also shows the relative contribution of the components of the diet to the total intake. Most of the estimates published in the literature have been made by this technique. The data quoted in Table 4 shows mainly estimates for persons consuming an average diet but the data from the NAS publication (1981) illustrates clearly the possible effects of extreme types of diet on the range of nitrate and or nitrite intake.

The third approach is crucially based on the relationship of dietary intake and urinary excretion of nitrate. Chivers et al, (1984) used nitrate excretion as a direct measure of total dietary intake. A later paper (Caygill et al, 1986) applied a correction factor such that dietary intake was equal to 1.5 x the urinary nitrate.

The range of dietary nitrate intakes observed for average diets ranges from 31.4 mg NO_3^-/person/day for Norway (Gislason and Dahl, 1980) to 130 mg NO_3^-/person/day for Poland (Nabrzyski and Gajewska, 1984), (table 4).

Of the data quoted in Tables 3 and 4 the estimates from the NRC (1981) committee are probably the most soundly based, certainly the best documented. The observed values by Chilvers et al (1984) relate to persons consuming water with high nitrate levels. Closer examination of the available data shows that the main source of nitrate in the average diet is vegetables. This is demonstrated in Table 5 where vegetables are shown to provide on average 82% of dietary nitrate. In the case of a vegetarian the total amount of nitrate ingested daily could approach 300 mg.

It must be stressed that measured and calculated daily intake values are averages. As the main source of nitrate is vegetable and the different sorts of vegetables vary in their nitrate content, and as vegetables consumption by an individual varies meal to meal, the daily variation in nitrate intake is considerable.

Where nitrate levels in the water supply exceed 50 mg NO_3^- l^{-1} nitrate from water is likely to be the major single source of the total nitrate intake. The contribution of water nitrate to total nitrate intake is shown below :

Water NO_3^- conc. (mg l^{-1})	Daily NO_3^- intake mg NO_3 from		Proportion derived from water (%)
	Water	Food	
10	14	57	20
50	71	57	55
75	107	57	65
100	143	57	71
150	214	57	79

(modified from UK, DOE, 1979)

Chilvers et al, (1984) studied the nitrate intake of 404 persons consuming well waters and reached the same conclusion.

Neonates represent a special case. Breast fed infants have a low nitrate intake, whilst those given infant formula feeds receive nitrate from the water. The liquid intake of neonates and young infants is generally about 150 ml/kgbw/d (Hull and Johnstone, 1987).

The daily intakes of nitrite (Table 4) also show a wide range from 0.34 mg/person/day in the US (FDA) to 11.2 mg NO_2^-/person/day also from the US (White, 1975, 1976). This compares with the WHO maximum recommended level of 8 mg NO_2^-/day.

By contrast, the major source of nitrite in the average diet is cured meat products. Further small amounts also come from some fish and cheese products that have nitrite added during processing. Nitrite does also arise in small quantities from vegetables and cereal products, almost certainly the result of changes taking place during storage, food preparation and cooking.

Nitrate and nitrite may also be formed by absorption of inhaled nitrogen oxides (WHO, 1984b). It has been estimated that absorption of all nitrogen oxides by an adult man in a polluted area would produce an intake of approximately 0.4 mg NO_3^-/day.

4.2 Endogenous Nitrate Synthesis

Studies in man and animals have shown that nitrate can be formed by mammalian metabolic processes. Quantification of endogenous nitrate synthesis is an area of active research and is of great importance in assessing total exposure to nitrate. The studies on this subject are summarized below.

4.2.1 Animal Studies

4.2.1.1 In vivo

Green et al (1981a) varied the dietary nitrate content of germfree and conventional rats and measured the nitrate excretion. This was in excess of intake in the low nitrate group (<0.06 mg/day) and less with the high nitrate group (0.93 mg/day). In addition, the diet was supplemented with $Na^{15}NO_3$. About 33 to 50% of the label was excreted as nitrate in both the conventional and germfree rats. In conventional rats, an average of 16% of the ingested label appeared as $^{15}NH_4^+$ and ^{15}N-urea in the urine. Although about 40% of ingested nitrate (0.06 mg daily) was expected to be excreted in urine, rats excreted about 0.37 mg nitrate daily. This excess is a clear indication of endogenous synthesis of nitrate at a rate of about 1.24 mg/kgbw/day by the rat.

Dull and Hotchkiss (1984a) performed nitrate balance studies in the ferret in the same way. Excretion of nitrate was lower than ingested when oral doses of nitrate were higher than 0.39 mg/day. The excretion exceeded intake when nitrate ingestion levels were less than 0.39 mg/day. Only 36% of ^{15}N-labelled nitrate was excreted as ^{15}N-nitrate in the urine. In addition, oral doses of ^{15}N-labelled ammonia resulted in the incorporation of ^{15}N into nitrate in urine and faeces. The net biosynthesis of nitrate in the ferret was demonstrated to be 0.55 to 0.64 mg/kgbw/day. A 200% increase in nitrate excretion in the urine was observed after treatment with neomycin sulfate and mycostatin to reduce the gut microflora population.

Wagner et al (1983a) measured ^{15}N-nitrate in urine 24 hours after dosing 6 rats orally with 154 mg ^{15}N-ammonium acetate (37 mg NH_3). After one week the same dose of ^{15}N-ammonium acetate plus 1 mg E. coli lipopolysacharides (LPS)/kgbw were administered intra-peritoneally to the same 6 rats. On average, the urinary ^{15}N-nitrate increased by a factor 20 following LPS treatment (LPS is an endotoxin which induces fever). Two other types of inflammatory agents produced changes in nitrate biosynthesis. Intraperitoneal Carrageenan 150 mg increased urinary nitrate excretion by 200% and subcutaneous turpentine 0.5 ml caused a delayed increase of urinary nitrate excretion of 300% after 3 days.

Subsequently Wagner et al (1985) studied the precursor of the biosynthesized nitrate. He continuously infused rats for 96 hours with ^{15}N-ammonium acetate. The nitrogen in the excreted nitrate was 36% labelled with ^{15}N, the percentage conversion of total NH_3 into nitrate was 0.007%. The authors conclude, that approximately 50% of endogenous nitrate is derived from other as yet unidentified sources.

Saul and Archer (1984) administered by gavage 53.5 mg ^{15}N-ammonium chloride to rats daily during 5 days. Ammonia was converted to nitrate with a yield of 0.008%. Further, it was estimated that a 215 g rat produces 0.19 mg nitrate per day via ammonia oxidation (equivalent to 0.87 mg/kgbw/day).

Stuehr and Marletta (1985) studied E. coli lipopolysaccharide stimulated nitrate synthesis in LPS-sensitive C3H/He and LPS-resistant C3H/HeJ mice. Intraperitoneal injection of 15 µg of LPS led to a temporary 5- to 6-fold increase in the blood nitrate concentration in the LPS-sensitive mouse strain but not in the LPS-resistant strain even at an i.p. dose of 175 µg LPS. Mycobacterium bovis injection into mice of both strains resulted in a large increase of nitrate production over the course of the infection and increased the urinary nitrate excretion from 0.12 mg/day to 5.6 mg/day (3.6 mg/kgbw/day to 164 mg/kgbw/day on the 9th day after injection).

4.2.1.2 In vitro

Dull and Hotchkiss (1984 b) studied the conversion of NH_3 into NO_3^- by rat liver. A xanthine-xanthine oxidase reaction oxidised NH_3 in the presence of H_2O_2. An in vivo dosed inhibitor of superoxidedismutase enhanced the in vitro conversion of NH_3 to NO_3^-. Thus intracellular oxidation by activated oxygen seems to be involved in endogenous nitrate formation.

Stuehr and Marletta (1985) observed that peritoneal macrophages derived from an LPS-sensitive mouse strain C3H/He produced nitrite and nitrate (3:2 M/M) in the presence of LPS (10 µg/ml) in vitro. T-lymphocytes enhanced this production. No nitrite or nitrate production was observed in macrophages of the LPS-resistant mouse strain C3H/HeJ. In a subsequent study Stuehr and Marletta (1986) observed in the same cultures of macrophages that addition of gamma-interferon alone or gamma-interferon and LPS at the same time, led to a higher nitrite and nitrate production than the addition of LPS alone.

4.2.2 Human Studies

4.2.2.1 Dietary nitrate balance studies

Tannenbaum et al (1978) determined the urinary nitrate excretion of 6 male volunteers between the age of 68 to 72 who received a standardized diet with a low, known nitrate content. The urinary nitrate excretion (ca 62 mg/day) clearly exceeded the dietary nitrate intake (0.62 mg/day).

Green et al (1981b) repeated the above diet study with young healthy students and found the same excess urinary nitrate excretion.

Bartholemew and Hill (1984) also performed diet studies in healthy volunteers and in patients with an ileostomy. They found that excretion of nitrate and nitrite in faeces or ileostomy fluid was less than 2% of the daily intake. There was 20 mg/day excess of

urinary nitrate excretion over dietary intake-lower than the 40 mg urinary excess observed by Tannenbaum et al (1978) and Green et al (1981b). Bartholomew and Hill (1984) concluded that endogenous nitrate synthesis is low compared to normal dietary sources (20 mg versus 100 mg). Further, they raised doubt about the accuracy of the nitrate determinations of the diet in nitrate balance studies.

A recent dietary balance study (Lee et al, 1986), using a more accurate method for nitrate analysis, demonstrated a consistent excess of urinary nitrate over ingested nitrate/nitrite by an average of 54 mg/day, equivalent to about 1 mg NO_3/kgbw/day. The average daily urinary nitrate excretion was 60 to 148 mg/day.

All four papers demonstrate that at low nitrate intake levels the excretion of nitrate exceeds nitrate intake, suggesting extensive synthesis. This is supported by Tannenbaum, Green and Lee, whilst Bartholomew and Hill believe that inaccuracies of nitrate analysis make endogenous synthesis appear larger than it really is.

4.2.2.2 Enhancement of endogenous nitrate synthesis during infectious disease.

That nitrate can be excreted in excess as a result of intestinal infections was recorded by Mayerhofer (1913) who noted that nitrate could not be found in the urine of healthy breastfed infants but even minor intestinal upsets quite often gave rise to nitrate positive urine samples. Subsequently Catel and Thunger (1933) found that infants fed only human milk excrete nitrate in noticeable quantities. Excretion was attributed to endogenous synthesis; daily excretion varied greatly in the 10 infants studied. At that time only semi-quantitative measurements could be made but the authors did note increased nitrate excretion by one infant suffering from enteritis.

Wagner and Tannenbaum (1982) observed an increase in urinary nitrate excretion from 43 mg to 280 mg NO_3^- per day in a human subject on a low, constant intake of dietary nitrate of 7.4 mg/day, when he developed a non- specific diarrhoea. The authors concluded

that activation of the reticuloendothelial system significantly increased nitrate biosynthesis and suggested that it might be dependent upon oxygen radical formation.

Similar findings have been described by Hegesh and Shiloah (1982). They determined nitrate, nitrite and methaemoglobin levels in blood and the urinary nitrate excretion in 58 hospitalized infants with acute diarrhoea. 130 subjects of different age groups with no gastrointestinal disorders acted as controls among these were 30 infants. Although the control and diseased infants were given the same low nitrate content food preparations (2-7 mg NO_3^- per infant per day), the nitrate level in blood was 7 times higher and the urinary nitrate excretion 15 times higher in the diseased than in the control infants. Subsequent evaluations of the data by the Task Force revealed daily urinary nitrogen excretion to be 1 mg NO_3^-/kgbw/day for control infants and 15 mg NO_3^-/kgbw/day for infants affected by diarrhoea. Twelve of the diseased infants had a methaemoglobin level of more than 8% and a mean blood nitrate of 37 mg NO_3^- per liter. The authors summarised their clinical experience with methaemoglobinaemia as follows :

> "Infantile methaemoglobinaemia is of much more frequent occurrence among hospitalized newborns and young infants than is generally appreciated. When present, acute diarrhoea of different etiologies is almost exclusively the cause of hospitalization. No correlation between this condition and ingestion of food or water containing high concentrations of nitrates or nitrites was found. This study demonstrates for the first time that high blood nitrates are a regular part of the syndrome. The evidence presented is interpreted as indicating that diarrhoea results in endogenous, de novo synthesis of nitrites, and this is the principal cause of infantile methaemoglobinaemia".

Wettig et al (1987) described a 25 year old pregnant patient with Lambliasis and a pathogenic E. coli infection with diarrhoea. Her nitrate intake was less than 30 mg/d but she had nitrate concentrations in morning saliva and urine of 700-900 mg l^{-1} over 6 days. When the Giardia lamblia were eliminated by treatment with

metronidazol the nitrate concentrations fell to a normal level of less than 100 mg l^{-1}. Other patients similarly infected had elevated nitrate concentrations in urine, though not so extreme (149 mg/l before treatment, 54 $\pm$ 40 mg/l after treatment). In all these cases no clinical methaemoglobinaemia was present.

4.3 Summary

Nitrate is a normal constituent of foodstuffs.

Most of the dietary nitrate is from vegetables. These vary greatly in nitrate content, depending on type, variety and growing conditions.

Dietary nitrate intake ranges between 30 and 300 mg NO_3^-/day; it is difficult to measure accurately and varies with individual dietary habits. The nitrate intake of vegetarians is considerably higher than non-vegetarians.

At a water nitrate concentration of approximately 50 mg l^{-1} drinking water and food both contribute about equally to the daily nitrate intake.

For infants given infant formula feeds the water can often be the main source of dietary nitrate. Milk itself contains less than 5 mg NO_3^- l^{-1}.

Nitrate synthesis occurs in animals and man but human nitrate balance is experimentally difficult to measure; the current estimate of the endogenous synthesis is about 54 mg/day. The endogenous synthesis of nitrate can be substantially increased during gastrointestinal infection.

Nitrite intakes reported in the literature range between 0.34 to 11.2 mg/d. The major source is cured meat.

5. FATE OF NITRATE IN THE BODY

5.1 Absorption and Excretion

5.1.1 Studies in Animals

In laboratory animals nitrate is absorbed from the proximal small intestine and becomes distributed throughout the body after about 60 minutes indicating rapid absorption (Balish et al, 1981). About 60-70 % of an oral dose is excreted in urine (Wang et al, 1981). Nitrate does not reach high concentrations in the saliva since the active transport system responsible is not found in the rat, unlike man (Hartman, 1982). Lin and Lai (1982) detected nitrate (0.5-2 ppm) in the small and large intestine of rats fed a diet containing 5000 ppm nitrate. Rats, unlike human beings, may actively secrete nitrate into the lower intestinal tract (Hartman, 1982; Witter & Balish, 1979).

Using (^{15}N) nitrate, Schultz et al (1985) showed that rats with conventional flora excreted 54% of orally administered nitrate unaltered in the urine; 11% of the label was found as urinary ammonia and urea leaving 35% unaccounted.

5.1.2 Studies in Man

Nitrate is rapidly absorbed from the proximal small intestine (Hartman, 1982; Bartholomew & Hill, 1984) and is distributed throughout the body. Nitrate excretion in saliva and sweat reaches a peak 3-6 hours after exposure and approximately 60-70% of an oral nitrate dose is excreted in urine in the first 24 hr (Selenka, 1983; Bartholomew and Hill, 1984), a shorter period than in the rat. In man blood nitrate is transferred to saliva by an active transport system shared by iodide, thiocyanate and nitrate. It is estimated that approximately 25% of ingested nitrate is secreted in saliva (Spiegelhalder et al, 1976; Tannenbaum et al, 1976).

It is likely that nitrate enters the large bowel of man from the bloodstream although only low levels of nitrate and nitrite have been detected in human faeces (Saul et al, 1981; Wagner et al, 1983b). This

is consistent with the extremely rapid reduction of nitrate by faecal microorganisms and the high reactivity of nitrite (Saul et al, 1981; Archer et al, 1981; Wise et al, 1982; Balish et al, 1981).

In a study in which twelve humans received an oral dose of (^{15}N) nitrate, about 60% appeared in the urine as nitrate and 3% as ammonia or urea, with smaller amounts (0.3%) of (^{15}N) being found in faeces leaving about 37% unaccounted (Wagner et al, 1983b).

5.2 Reduction of Nitrate to Nitrite

In vitro and in vivo studies show that nitrate can be reduced to nitrite by bacterial and mammalian metabolic pathways; nitrate reductase activity is possessed by a wide range of micro-organisms including many present in the mammalian gastrointestinal tract (Payne, 1973) .

Bacteria can also further reduce this nitrite to ammonia which is subsequently transformed and into urea and amino acids. (^{15}N) nitrate studies in rats and man indicate that this occurs in vivo (Schultz et al, 1985; Green et al, 1981a) although in vitro studies show that the further reduction of nitrite by enteric bacteria is slower than the production of nitrite so that this accumulates (Wise et al, 1982).

Nitrate reduction has been detected in the gut mucosa of germ free rats although the reaction was much slower than in the gut contents (Ward et al, 1986).

The widespread occurence of nitrate reductase activity in bacteria means that nitrite is formed where large numbers of bacteria are found, namely the mouth, the stomach (if the gastric pH is >5), the distal small intestine and the colon, the infected urinary bladder and the vagina. The amount of nitrite formed depends on the number, types and activity of the bacteria present and the availability of nitrate.

About 25% of ingested nitrate in man is secreted in saliva and of this 20% reduced to nitrite by the bacteria, thus about 5% of dietary nitrate is converted to nitrite in saliva. Saliva is therefore a major site of

nitrite formation in man (Spiegelhalder et al, 1976; Walters & Smith, 1981).

Salivary nitrate and nitrite concentration are roughly proportional to the amount of nitrate ingested (Spiegelhalder et al, 1976; Bartholomew & Hill, 1984). Fasting levels of salivary nitrite are about 5-10 mg/l (Spiegelhalder et al, 1976; Tannenbaum et al, 1976) although these can rise to 150 mg/l after ingestion of a nitrate rich meal containing 470 mg NO_3. It has been estimated that after such an intake 40-70 mg nitrite could enter the stomach (Ishidata et al, 1975; Spiegelhalder et al, 1976). Spiegelhalder et al reported that ingestion of less than 54 mg nitrate did not alter salivary nitrate and nitrite levels, although this is not a consistent finding (Walters and Smith, 1981; Stephany and Schuller, 1980). There appears to be considerable individual and diurnal variation in nitrate/nitrite levels in saliva (Tannenbaum et al, 1976; Bartholomew & Hill, 1984).

Since the normal human stomach is virtually sterile little or no reduction of nitrate occurs and nitrite levels found in the stomach are usually low. Where gastric pH is elevated colonisation by bacteria occurs and nitrite levels in gastric juice increase (Table 6). A direct correlation between gastric pH, bacterial colonisation and gastric nitrite concentration has been noted in healthy populations with a range of gastric pH values from 1 to 7 (Mueller et al, 1983; 1986) and in individuals with gastrointestinal disorders (Ruddell et al, 1976), pernicious anaemia or hypogammaglobulinaemia (which is usually accompanied by achlorhydria). Gastric nitrite levels in such conditions can reach 6 mg l^{-1} (Ruddell et al, 1976; 1978; Dolby et al, 1984).

Nitrate reduction may also occur in the stomach and small intestine of newborn infants, depending upon the flora that has been established, the amount of hydrochloric acid secreted, and the feeding rhythm. It is often stated that the stomach of infants is not acidic and not sterile and thus presents favourable conditions for nitrate reduction to nitrite. However, Agunod et al (1969) found only one infant with achlorohydria in 12 infants studied (ages 12 hours to 3 months; 33 samples of gastric juice) Simon et al (1962) found in 7 infants less than 7 months old their

gastric acid secretion was sufficient to prevent significant bacterial growth in the stomach during 0.5 - 3 hours after feeding. Bodo (1955) described 7 cases of infant well-water methaemoglobinaemia. In 6 of 7 infants aged between 2 and 10 weeks of age the gastric pH was found to be 2.5; 3.5; 4.5 and 3 with pH 5.0 and the stomachs were sterile. In one of the 7 E. coli was found in the stomach. Enterobacteria were present in the duodenum, a characteristic also noted by Barna et al (1974). Thus even in infants the stomach conditions do not usually favour microbial growth and nitrate reduction. Since symptoms of nitrite intoxication appear some time after nitrate ingestion (Amundsen, 1948; Cornblatt and Hartmann, 1947) the main area of intestinal nitrate reduction may not be the stomach; the contribution of duodenal nitrate reduction is not known.

5.3. Transformation of Nitrite to Nitroso-compounds

The conversion of nitrate to nitrite and then to nitroso-compounds has lead to an interest in the role of these processes in the aetiology of human cancer. Small quantities of N-nitrosocompounds are formed in the body and are of concern because many are highly carcinogenic and mutagenic. Both N-nitrosamines and N-nitrosamides induce tumours in a wide variety of tissues in many animal species and at extremely low exposure levels (Montesano and Bartsch, 1976 ; Schmähl and Habs, 1980 ; Bogovski & Bogovski, 1981 ; Archer 1982 ; Lijinisky 1984a, b ; Peto et al, 1984). Although there is no direct evidence that N-nitrosocompounds cause human cancer, it is unlikely that man is insensitive to their effects. Even so the incidence of gastric cancer continues to diminish both within Europe and the USA suggesting that this tumour may not be associated with nitrate ingestion.

Nitrite, in the form of nitrous acid, reacts with secondary and tertiary amines to yield nitrosamines, secondary and tertiary amides to form nitrosamides and N-substituted ureas and carbamates to yield nitrosoureas and nitrosocarbamates. These reactions and reactions with phenols, alcohols and thiols have been reviewed extensively (Archer, 1982; Hartman, 1982; Green and Tannenbaum, 1982 ; Challis et al, 1987 ; Challis, 1985). The reaction of nitrous acid with secondary amines is pH dependent. The maximum rate is at pH 2.5-3.3 (Mirvish, 1975; Challis, 1985) and is proportional to the concentration of the unprotonated amine

so that weak bases are more rapidly nitrosated than strong bases (Archer, 1982). The rate is also related to the square of the nitrite concentration although at low nitrite concentrations such as occur in human stomach, nitrosation of amines may be 1st order with respect to nitrite (Challis et al, 1982). Amides and ureas are more rapidly nitrosated with decreasing pH (Challis, 1981). In the normal human acidic stomach nitrosation of aromatic nitrostable substances may predominate over N-nitrosation because of their relative abundance (Challis, 1985). Such compounds, along with ascorbic acid and bisulphite act as nitrite scavengers and effectively block N-nitrosation (Mirvish et al, 1972 ; Challis, 1973). On the other hand thiocyanate, halides and surfactants can accelerate N-nitrosamine formation (Archer, 1982) although the formation of N-nitrosamides, nitrosoureas and nitrosoguanidines is not catalysed by such substances (Challis, 1981, 1985).

Bacteria can influence nitrosation by lowering pH and they may also nitrosate secondary amines enzymically at neutral pH (Suzuki & Mitsouka, 1984 ; Leach et al, 1985); the kinetics of this reaction differs markedly from those for acid-catalysed nitrosation (Leach et al, 1985). Bacterial nitrosation means that the process may not be confined to the acid stomach but (as with nitrate reduction) may also occur at other sites which harbour bacteria.

5.4 Sites of Formation of Nitroso-compounds

5.4.1 Saliva

N-Nitrosocompounds are formed in vitro in saliva using incubations (Tannenbaum, 1977; Hart & Walters, 1983), but the unfavourable neutral pH and short residence time limit the amounts formed in vivo. Although Hart & Walters (1983) found compounds corresponding to nitrosamines and nitrosamides in saliva and Ellen et al (1982b) detected dimethylnitrosamine in saliva, the amounts were unaffected by higher intakes of nitrate.

5.4.2 Stomach

The acid gastric juice ensures that the stomach contents are virtually

sterile except for a few hours immediately after a meal and no bacterial reduction of nitrate can occur. Any gastric nitrosation results from reactions with ingested or salivary nitrite. Nitrite occurs at very low concentrations 0.05-0.6 mg l^{-1} (Walters et al, 1979; Kyrtopoulos et al, 1985) in part because it reacts rapidly with stomach contents. After a meal containing 38 mg NO_2^- kg^{-1} gastric nitrite rose to 14 mg NO_2^- l^{-1} after 45 min and fell rapidly after 60 min, cf. Table 6, (Walters et al, 1979).

The formation of nitroso-compounds has been detected in vitro and in vivo in man and laboratory animals. Since the rat and mouse have less acid stomachs than man and a resident bacterial population, studies in these species are probably more relevant to the hypochlorhydric than the normal human stomach.

5.4.2.1 Nitrosation in the Stomach, In vitro studies

In most in vitro studies on nitrosation human gastric juice has been incubated with nitrite and nitrosable compounds. In some studies the abnormally high nitrite levels (eg. Sen et al, (1969) used 5-10x the level seen after meals) may have effected the reaction kinetics (Challis, 1981), making the findings of doubtful relevance to man.

Walters et al (1979) incubated slurries of food (luncheon meat, eggs and milk) with human gastric juice and lower nitrite concentrations (21 mg l^{-1}) at pH 2 and found about 7mg nitroso-piperidine/kg food after 15 min with no further increase up to 3 hours. Groenen et al (1982), incubated a wide range of food products for 2 h at 37°C at pH 3 with lower levels of nitrite (5-7 mg l^{-1}) and were unable to detect volatile nitrosamine formation in most foodstuffs including vegetables, fruit, cerals, meat, dairy products and beverages. Low amounts (0.1-0.2 µg of nitrosamines) were formed with smoked sausage and cinnamon, and larger amounts (median 0.65 µg dimethylnitrosamine per portion) with fish; it was speculated that this was due to the high amine content of fish.

Kyrtopoulos et al (1985) studied nitrosation in fasting gastric juice over a range of pHs (2-7) and nitrite concentration (0.2-4.6 mg l^{-1}). The total N-nitroso-compounds formed from precursors naturally present in the juice was low (< 1.5 μM) at all pH's except 2 where up to 8 μM total N-nitrosocompounds were formed. The pH suggests that nitrosamides rather than nitrosamines were formed. Alam et al (1971) incubated piperidine with nitrate in the presence of rat stomach contents and detected nitrosopiperidine. Presumably the resident flora reduced the nitrate to nitrite and possibly catalysed the nitrosation reaction.

5.4.2.2 Nitrosation in the Stomach. In vivo studies

Administration of nitrite and nitrosatable compounds to rats and mice results in the presence of nitrosamines in gastric contents (Lijinsky and Greenblatt, 1972); exposure of animals to amines and nitrate has led to less consistent demonstration of nitrosation (Hashimoto et al, 1976).

N-nitroso-compound formation in the human stomach is also inconsistent and it would appear that there is considerable variation in the nitrosating capacity of individuals. Groenen et al (1985) detected dimethylnitrosamine in gastric juice 0.5-2 hours after consumption of meals with various nitrate contents. Diethylnitrosamine was occassionally detected but not other nitrosamines. The extent of nitrosation was consistent with in vitro nitrosation of foods (Groenen et al, 1985). Walters et al (1979) occasionally detected N-nitrosopiperidine in gastric contents of volunteers given meals containing nitrite (21-35 mg l^{-1}). In contrast, Lakritz et al (1982) found little or no change in the dimethylnitrosamine levels of gastric juice of 21 individuals after consuming meals of fish, beef or bacon together with nitrate-rich vegetables.

5.4.2.3 Nitrosation in gastric juice of hypochlorhydric individuals.
Variation in pH of the gastric juice may account for the high interindividual variation in gastric nitroso-compound formation.

Although the gastric pH of achlorhydrics is not conducive to chemical nitrosation of amines or amides the bacteria present may catalyse nitrosation (O'Donnell et al, 1987; Calmels et al, 1987). Stockbruegger et al (1984) detected higher levels of nitrosamines in the gastric juice of pernicious anaemia patients (22 µg/l) than in patients with untreated peptic ulcer (14 µg/l). Patients with atrophic gastritis also show elevated nitrite (8.4 mg l^{-1}). and nitrosamine levels in gastric juice (Schlag et al, 1982).

In a survey of fasting gastric pH, nitrite, nitrosamines and bacterial flora in a large number of subjects with various gastrointestinal disorders, Reed et al (1981) demonstrated a significant relationship in pernicious anaemia patients between N-nitrosamine concentration and pH ranging from 0.1 µmol/l at pH 1-1.5 to a mean of 1.2 µmol/l at pH 6.5-9.0 .

There is evidence of increased incidence of gastric dysplasia and gastric cancer in patients with pernicious anaemia, hypogammaglobinaemia, atrophic gastritis and gastric resection (Jones et al, 1978; Siurala et al, 1959; Hermans et al, 1976; Mosbech and Videbaek, 1950). Since hypochlorhydria is common to all these conditions there is a theory that gastric cancer may be related to the increased nitroso compound formation (Correa et al, 1975; Hill et al, 1973).

Patients on H_2-blocking drugs such as cimetidine and ranitidine which inhibit gastric acid secretion might be considered candidates for increased gastric nitrosation (Stockbrugger et al, 1982; Reed et al, 1981). However, eight healthy volunteers on cimetidine showed no significant increase in bacteria, nitrate, or nitrosocompound concentration and only minor effects on pH (Milton-Thompson et al, 1982).

Although nitrosocompound concentrations have been shown to be higher in the stomach in those with certain gastrointestinal diseases, excretion of nitrosoproline in urine, a qualitative indicator of in vivo nitrosation, is not elevated in patients with

gastric disease or surgery (Elder et al, 1984; Bartsch et al, 1984; Houghton et al, 1986, Hall et al, 1987).

5.4.3 Small intestine

Nitroso-compound formation in the small intestine has not been studied.

5.4.4 Colon

Bacteria taken from the large bowel of the rat and human faeces catalyse the formation of nitrosamines from nitrite or nitrate and amines at neutral pH in vitro (Klubes et al, 1972; Hill & Hawksworth, 1971; Suzuki & Mitsuoka, 1984). Analysis of faeces for nitrosocompounds is difficult since the compounds are prone to develop during the analytical procedures. Wang et al (1978) reported the presence of dimethyl and diethyl nitrosamine in human faeces. Hashimoto et al (1976) detected dimethylnitrosamine in caecal contents of rats pretreated with nitrosamine-synthesizing bacteria and fed dimethylamine and nitrate. Subsequent studies (Archer et al, 1981; Eisenbrand et al, 1981; Lee et al, 1981) failed to detect volatile nitrosamines in faeces in healthy individuals. The presence of nitrosamines in faeces of Japanese subjects (Suzuki & Mitsuoka, 1981) was later found to be an artefact (Suzuki & Mitsukoa, 1985).

5.4.5 Bladder and Vagina

The organisms commonly encountered in an infected bladder, E coli and Proteus spp are able to reduce nitrate, which may be present at concentrations up to 130 mg l^{-1} (Hartman, 1982; Hill & Hawksworth, 1971). In urinary tract infections 9-78 mg/day nitrite could be detected (Radomski et al, (1978), Brooks et al (1978), Hicks et al (1977), and Ohshima et al (1987) reported N-nitroso compounds in infected human urine. Although the normal vaginal flora has little capacity for nitrate reduction, chronic infective organisms (e.g. Trichonomas vaginalis) possess nitrate reductase activity and N-nitrosocompounds have been detected in vaginal exudate from some patients (Allsobrook et al, 1975).

5.4.6 Macrophage System

Nitrite is an intermediate during oxidation of ammonia to nitrate (see section 4.2). Therefore reaction of secondary amines with de novo synthesized nitrite to form nitroso-compounds should be considered.

Stuehr and Marletta (1985) observed that peritoneal macrophages in vitro, when simulated by lipopolysaccharides (LPS) of E. coli, produced nitrite and nitrate in a ratio of 3:2. When secondary amines were added to the test systems and LPS or interferon used to stimulate the macrophages, nitrosamine production was strongly increased and it paralleled the nitrite production (Miwa et al, 1986). However, addition of nitrite to the test system did not increase nitrosamine formation at the pH of the test culture of 7.4 suggesting that an activated stage of oxidized nitrogen is necessary for nitrosamine formation.

The experiments point to the potential contribution of macrophages to the endogenous formation of nitrosamines.

5.5. Nitroso-compound Formation and Dosimetry

Assessment of the extent of nitrosocompound formation in the body is fraught with difficulties and the various methods used have limitations.

5.5.1 Nitrosamines in blood

In blood, measurements have been confined to volatile nitrosamines; these are usually rapidly metabolised making estimates of rates of formation inaccurate.

Human blood levels of dimethyl- and diethylnitrosamines are low (0.1-2.5 µg/l) during fasting (Dunn et al, 1986), reflecting the level of their endogenous synthesis. When individuals were fed meals rich in amines and nitrate/nitrite, little or no increase in nitrosamine levels occurred (Lakrtiz et al, 1982; Gough et al, 1983; Fine et al, 1977; Melikan et al, 1981; Kowalski et al, 1980; Yamamoto et al, 1980; Groenen et al, 1985). Furthermore, Ellen et al (1982) found no

increase in volatile nitrosamines in blood or urine of 23 individuals who were given 2.5-9.0 g ammonium nitrate daily.

5.5.2 Nitrosocompounds in urine

Volatile nitrosamines.

Urinary nitrosamine levels remained unchanged after volunteers consumed meals of fish or beef with vegetables containing nitrate and nitrite (Lakritz et al, 1982). Ellen et al (1982) did not detect volatile nitrosamines in urine after ingestion of large quantities of ammonium nitrate. Urinary nitrosopiperazine has been detected after exposure to oral piperazine (Bellander et al, 1985, 1987; Spiegelhalder and Preussmann, 1984). In urinary tract infection, nitrosamines may be found in urine presumably as a consequence of bacterial reduction of urinary nitrate to nitrite and subsequent bacterial catalysis of nitrosation of urinary amines (Hicks et al, 1978).

Nitrosamino acids.

The principal method used to study nitrosation reactions in vivo in laboratory animals and man involves determining urinary excretion of certain N-nitrosoamino acids, notably nitrosoproline (Ohshima et al, 1982; Wagner et al, 1982) and nitrosothioproline (Tsuda et al, 1983; Ohshima et al, 1984). Most work has been done on nitrosoproline, a stable compound which is quantitatively excreted in urine.

The basal excretion rate of nitrosoproline (i.e. in people on a low nitrate diet and not given a dose of nitrite/nitrate or proline) is 2-7 µg/day (Wagner et al, 1984; Bartsch and Montesano, 1984; Oshima and Bartsch, 1981). Ingestion of high doses of proline and nitrate at the same time increases N-nitrosoproline excretion markedly. Thus 500 mg proline with vegetable juice containing 325 mg nitrate increased nitrosoproline excretion to 16-30 µg/day. This has to be placed in the context of the variation of nitrosoproline excretion in man ranging from 0 to 400 µg/day (Bartsch et al, 1984; Elder et al, 1984). When less than 195 mg nitrate was ingested, little or no increase in nitrosoproline excretion was seen (Ohshima and Bartsch, 1981).

Nitrosoproline excretion was also decreased by beer (Pignatelli et al, 1983) possibly due to its content of polyphenolic antioxidants such as chlorogenic acid.

5.6 Summary

In man, nitrate is rapidly taken up into the blood from the small intestine and mainly excreted in urine.

Part of the ingested nitrate (usually about 25%) is recirculated by excretion in saliva.

Part of the nitrate is reduced to nitrite, mainly in the saliva where usually about 5 mg NO_2^-/day is formed. There may be considerable variations from day to day and from person to person.

Nitrite is a reactive molecule which participates in numerous reactions with food components in the stomach. It is absorbed and reacts with haemoglobin forming methaemoglobin.

The quantitative balance of nitrate and nitrite in the human body is at present unknown.

Nitrosation of dietary compounds by nitrite can occur in human acid gastric juice in vitro yielding, n-nitroso compounds with amines and amides. Many n-nitrosocompounds are carcinogenic in animals and also presumably in man.

In human subjects with normal gastric acidity N-nitroso compounds have been detected occasionally in gastric juice after ingestion of foods containing nitrosatable amines and nitrate.

Increased nitrite and nitroso-compounds levels have been detected in human subjects who secrete inadequate gastric acid. Such individuals are at increased risk of developing gastric cancer.

Man excretes some nitroso-compounds in urine (e.g. nitrosoproline). This excretion can be increased by simultaneous ingestion of high doses of

In the study of Wagner et al, (1985-b) the nitrate used was labelled with (^{15}N), this demonstrated that the excreted nitrosoproline was not totally derived from ingested nitrate; the (^{15}N) nitrosoproline in urine accounted for between 10 and 60% of total nitrosoproline synthesis. This observation is supported by studies in the rat which show that the nitrosation of (^{14}C) proline proceeded equally in the presence or absence of orally administered nitrate (Mallett et al, 1985). Further evidence was afforded by Dull et al, (1986), who dosed ferrets with high amounts of ^{15}N-nitrate (500 mg l^{-1}) in drinking water. Less than 0.5 % of ^{15}N was incorporated into the excreted nitrosoproline. In addition, Tannenbaum (1987) observed no relation between dietary nitrate uptake and nitrosoproline excretion in humans.

These findings would be consistent with a significant part of the excreted nitrosoproline originating from reaction between proline and endogenously formed nitrite from NH_3-oxidation in macrophages.

Much less work has been done using other nitrosoamino acids to monitor nitrosation. Although nitrosothioproline is also excreted in urine, there is not always a clear correlation between its excretion and that of nitrosoproline (Elder et al, 1984; Bartsch et al, 1984). Wagner et al (1984) however did report a six fold increase in nitrosothioproline excretion to 4 µg/day after ingestion of 217 mg nitrate.

5.5.3 Factors affecting nitrosoproline excretion.

Wagner et al.(1985) reported that ascorbic acid (2 g/day) or α-tocopherol inhibited the incorporation of (^{15}N) nitrate into nitrosoproline by 81 and 59% respectively. These vitamins had no effect on the basal nitrosoproline excretion (i.e. in the absence of administered nitrate and proline).

The endogenous formation of nitrosoproline is increased by thiocyanate (Oshima et al, 1982) which also stimulates nitrosation in stomach juice in vivo and in vitro. This may explain the 2 fold increase in the excretion of nitrosoproline by smokers (Ladd et al, 1984) given nitrate and proline; smokers have higher thiocyanate levels in saliva than non-smokers.

Nitrosoproline excretion was also decreased by beer (Pignatelli et al, 1983) possibly due to its content of polyphenolic antioxidants such as chlorogenic acid.

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The quantitative balance of nitrate and nitrite in the human body is at present unknown.

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In human subjects with normal gastric acidity N-nitroso compounds have been detected occasionally in gastric juice after ingestion of foods containing nitrosatable amines and nitrate.

Increased nitrite and nitroso-compounds levels have been detected in human subjects who secrete inadequate gastric acid. Such individuals are at increased risk of developing gastric cancer.

Man excretes some nitroso-compounds in urine (e.g. nitrosoproline). This excretion can be increased by simultaneous ingestion of high doses of

nitrate and proline. It is, however, at present unclear if this excretion is influenced by variations in normal daily nitrate intake.

Although N-nitroso compounds can be detected in several body fluids, evidence suggests that ingestion of nitrate does not significantly increase their concentration.

6. TOXICITY OF NITRATE AND NITRITE IN ANIMALS

6.1 Background

Despite the low toxicity of nitrate ions (WHO, 1985b ; NAS, 1978, 1981 ; UK DOE, 1979) an impression exists that the nitrate currently ingested by the public in water and food could constitute a significant health hazard. This paradox appears to stem from confusion between the effects of nitrate and of nitrite particularly by dubious logic which lays the effects of one material at the door of the other. The toxic effects induced by nitrate and by nitrite must be clearly distinguished. The toxic effects often ascribed to nitrate are in fact, the results of the action of its reduction product. Hence, when toxicological experiments and experiences with nitrate are evaluated, allowance must be made for possible variations and limitations produced by this bacteria-mediated reduction with the different types of animals and feed used.

6.2 Nitrate Toxicity in mammals excluding man

6.2.1 Acute Toxicity

In ruminants the nitrate is reduced because of the low redox potential in the rumen (Keck, 1984). Fatal methaemoglobinaemia may occur within a few hours with a dose of 160 mg NO_3^-/kgbw given to cows as nitrate rich hay. Methaemoglobin levels of 50% or more may result depending on the bacterial flora present within the rumen (Kemp et al, 1977).

In monogastric species the fatal dose of nitrate when administered by stomach tube is an order of magnitude higher than in ruminants. The cause of death in rodents may be disturbance of osmotic and electrolyte balance; death occurs usually within one hour (Heisler, 1979). The high salt concentration in the stomach makes reduction by microbial organisms to nitrite improbable.

The following oral LD 50-values were found:

Species	Nitrate Salt	LD_{50} oral mg/kgbw
rabbit	$NaNO_3$	2680
rat	KNO_3	3750
rabbit	KNO_3	1900
rat	NH_4NO_3	4820
rat	NH_4NO_3	2450

(NIOSH Registry Toxic Effects Chemical Substances, 1987).

6.2.2 Subacute toxicity

Maekawa et al (1982) fed groups of 10 male and 10 female F344 rats a diet containing 1.25, 2.5, 5, 10 or 20% nitrate ad libitum for 6 weeks. All females and 7 males in the 20% group died during the experiment. At autopsy the abnormal colour of the blood and the spleen due to methaemoglobin was marked in rats of the two highest dose groups. In all groups except the males given 20% and the females given 10% nitrate, there was less than a 10% decrease of body weight gain compared with controls. The maximum dose without adverse effects was 5% nitrate in the diet. This is equivalent with an estimated average dose of at least 2500 mg/kgbw/day.

These findings were confirmed by Til et al (1985), who fed groups of 10 male and 10 female Wistar rats during a period of 4 weeks two different basal diets, supplemented with 1, 2, 3, 4 or 6% KNO_3 or with 1, 2, 3, 4 or 5% $NaNO_3$. Their general condition, behaviour and survival were not adversely affected. A significant dose related increase was seen in the methaemoglobin levels in females receiving more than 2% dietary nitrate but the increase was always smaller than 1.5%. There was a dose related increase in the relative weight of the kidneys. The incidence of tubular nephrosis decreased with increasing levels of KNO_3 and also with 5% $NaNO_3$ in the diet. The diet containing 1% nitrate was without any adverse effect. This diet results in a daily intake of 500 mg/kgbw.

6.2.3 Chronic toxicity including carcinogenicity

Groups of 20 male and 20 female rats were fed a diet containing 0, 0.1, 1, 5 or 10% $NaNO_3$. Growth rate was decreased at the 5% and 10% dietary levels. Complete histopathological examination did not reveal adverse effects and tumour incidence was not raised in test groups. The highest no adverse effect level in this study was 1% equivalent with a daily uptake of 500mg/kgbw (Lehman, 1958).

Lijinsky et al (1973c) dosed groups of 15 male and 15 female rats with drinking water, containing 0 or 0.5% $NaNO_3$ during 84 weeks; the animals were sacificed 20 weeks later. The tumour incidence was not different in the two groups. A dose of 0.5% $NaNO_3$ in drinking water is equivalent to 500 mg/kgbw/day.

Groups of 10 male and 10 female Sprague-Dawley rats received drinking water containing 0 or 4000 mg $NaNO_3$ per litre (approximated daily dose 0 and 400 mg/kg for 14 months). The methaemoglobin levels of the groups were the same (Chow et al, 1980).

Maekawa et al (1982) administered groups of 50 male and 50 female F344 rats diet containing 0, 2.5 or 5% sodium nitrate for 2 years. The survival rate of nitrate dosed animals was significantly higher than that of the controls. No significant difference in the incidence of malignant tumours was seen and the maximum no adverse effect level was approximately 2500 mg/kgbw/day.

6.2.4 Reproduction

Sleight and Atallah (1968) administered groups of male and female guinea pigs drinking water containing 300, 3500, 10000 or 30000 mg KNO_3 l^{-1} for 143-204 days. Reproduction in females was considerably decreased at the highest dose level. Conception took place at all dose levels, so male fertility was apparently not affected. The concentration of 10,000 mg KNO_3 l^{-1} drinking water (corresponding to at least 500 mg KNO_3/kgbw/day) was the highest dose without adverse effect.

6.2.5 Mutagenicity

Nitrate did not show any mutagenic activity in microbial tests with Salmonella typhimurium and Escherichia coli under aerobic conditions. However, under anaerobic conditions nitrate was mutagenic to Escherichia coli, probably due to reduction of nitrate into nitrite (Konetzka, 1974).

Syrian golden hamsters were dosed on day 11 and 12 of pregnancy with 500 mg $NaNO_3$/kgbw. After 24 hours the foetuses were excised and processed to embryo cell cultures. Gene mutations, micronuclei and chromosomal aberrations were not found in these cultures (Inui et al, 1979).

KNO_3 and $NaNO_3$ were without mutagenic activity in the Ames test with several strains of Salmonella typhimurium (Ishidate et al, 1984). The same authors observed chromosomal aberrations in Chinese hamster fibroblast cell cultures exposed to $NaNO_3$ but not if exposed to KNO_3. The chromosome aberrations may have been caused by the high osmotic pressure of the $NaNO_3$ test medium (70 mM $NaNO_3$ versus 10 mM KNO_3), because NaCl (140mM) also produced chromosome aberrations in this test.

Groups of 8 mice were dosed twice by gavage at a 24 hour interval, with 79, 236, 707 or 2120 mg $NaNO_3$/kgbw. A small but significant increase of micronuclei was found at 79 and 236 mg/kgbw but not at the higher dose levels when cytoxic effects on the bone marrow were observed. Chromosomal aberrations were significantly increased at only 707 mg/kgbw. Groups of 4 rats which received the same treatment did not show chromosomal aberrations in the bone marrow. However groups of 6 rats, which received these doses daily for 2 weeks, showed a significant increase in chromosomal aberrations at all dose levels. That the observed effects were caused by metabolic products like nitrosamines and not by nitrate itself cannot be excluded (Luca et al, 1985).

6.2.6 Effects on the thyroid gland

Wijngaarden et al (1953) studied the effects of high levels of nitrate in drinking water on the iodide-concentrating mechanism of the thyroid gland. Nitrate appeared to compete with iodide in uptake by the thyroid, leading to a decreased iodide uptake. Höring et al (1985) and Seffner et al (1985) exposed rats to drinking water containing 40, 200, 1200 or 4000 mg NO_3 l^{-1} for 100 days. Thyroid function was examined by ^{131}I-uptake, ^{131}I-serum level, ^{131}I-incorporation into proteins, the thyroid gland weight and histopathological parameters. Thyroid weight and ^{131}I-uptake appeared to be changed slightly at all dosage levels. Histopathological parameters were altered at all dose levels but there was no dose-response relationship in the finding.

6.2.7 Acceptable daily intake of sodium nitrate

These toxicity data suggests a maximum no-effect level in rodents of 500 mg NO_3/kgbw. WHO (1974, 1980) also considered 500 mg $NaNO_3$/kgbw as the highest daily dose over lifetime without adverse effects in rats. On this basis, by application of a 100 X safety factor, the WHO/FAO gives as the acceptable daily intake (ADI) 5 mg $NaNO_3$ (3,65 mg NO_3^-/kgbw), equivalent to 219 mg NO_3^-/day for a 60 kg man.

This recommendation originally refers to the amount used as food additive, exclusive of "amounts naturally present in food" (WHO, 1974). However, it is also used by some authors in the sense of total daily intake of nitrate from all sources, thus introducing an ambiguity.

As this level of intake is the same or less that seen in large groups of people, eg vegetarians, and as nitrate is a normal human metabolite, the subject of ADI for intake of nitrate seems worthy of re-examination.

6.2.8 Conclusions

High doses of nitrate administered to rodents influence reproduction, growth, the level of methaemoglobin and relative kidney weight. The highest no adverse effect levels are given in Section 6.5.

Mutagenic activity *in vivo* of high doses of nitrate is difficult to evaluate because of the presence of nitrite produced by reduction of the nitrate.

6.3 Nitrite Toxicity in Mammals excluding Man

6.3.1 Acute toxicity

The oral LD 50 of sodium nitrite (NIOSH, 1987) was :

mouse	214 mg/kg
rabbit	186 mg/kg
rat	180 mg/kg

Nitrite dilates blood vessels throughout the body by directly relaxing the smooth muscles, resulting into a fall in blood pressure. It also causes methaemoglobinaemia.

6.3.2 Subacute and subchronic toxicity

Studies in which the nitrite was administered in the diet are difficult to evaluate since it reacts with food constituents, decreasing the level of nitrite present. Administration in drinking water is more reliable.

Maekawa *et al* (1982) gave groups of 10 male and female F344 rats 20 ml sodium nitrite solutions containing 0, 0.06, 0.125, 0.25, 0.5 or 1% during 6 weeks as a drinking water replacement. Four females in the 1% group and one male and one female in the 0.5% group died. Only in the 1% group was body weight gain depressed by more than 10%. Methaemoglobin was marked in rats of the 0.5% and 1% group. The highest no adverse effect level appeared to be 0.25% nitrite in

drinking water. This is equal to a daily dose of about 200 mg/kgbw/day.

Groups of 8 male Sprague-Dawley rats were allowed drinking water containing 0 or 200 ppm nitrite for 16 weeks. Blood samples were taken periodically. The methaemoglobin content of the exposed rats ranged from 0.5 to 3.1% as compared with 0 - 1.2% in the control group. Except for a higher incidence of pulmonary lesions (increased lung weight, micro-abscesses), no adverse effect was observed in the exposed compared to the control group (Chow et al, 1980).

6.3.3 Chronic Toxicity including carcinogenicity

Van Logten et al (1972) fed groups of 30 male and 30 female Wistar rats for 2 years a diet containg 40% canned meat and supplemented with 0.5% or 0.02% $NaNO_2$ with or without 1% glucono-delta-lactone. Observations included haematology, clinical biochemistry, histopathology, alpha-foetoprotoein in the serum and the DNA-content of the liver cell nuclei. There was no difference in incidence of any preneoplastic charge or tumour in treated and control animals.The highest actual nitrite level in the feed was 4000 ppm equivalent to about 200 mg/kgbw/day.

Gruener and Shuval (1973) exposed five groups of 8 male rats to drinking water containing 0, 100, 1000, 2000 or 3000 ppm nitrite for 24 months. The methaemoglobin levels (determined at midnight to overcome difficulties brought about by nocturnal behaviour of rats and the short half life of methaemoglobin) were increased by 5, 10 and 20% in the 1000, 2000 and 3000 ppm groups. The following pathological findings were observed more frequently in these groups :

- congestion of liver and spleen,
- focal inflammatory and degenerative changes in some kidneys,
- general emphysema, dilated bronchi, lymphocyte infiltration of bronchial epithelium in the lungs,
- pronounced degenerative foci in the heart and thin and dilated coronary arteries.

There were no differences in growth, survival and blood haemoglobin, glucose, pyruvate and lactate. The highest dose level without adverse effect was 100 ppm equivalent to a dose of 5-10 mg/kgbw/day.

Pregnant Sprague-Dawley rats and Syrian golden hamsters were fed from the time of conception a diet containing a measured average concentration of 263 ppm but a nominal concentration of 1000 ppm. Young were randomly selected from the F1 generation, for carcinogenicity studies on rats or hamsters. In rats an F2-generation was derived from the F1. The studies were terminated in F2 rats at week 125 and in hamsters at week 100. There was no difference between nitrite exposed and control animals in survival and tumour incidence except for an increase in incidence of lymphoreticular tumours in rats (Shank and Newberne, 1976).

Newberne (1979) repeated this study. Pregnant rats received a diet or drinking water, containing 0, 250, 500, 1000 or 2000 ppm (nominal levels) of $NaNO_2$ 5 days before they gave birth to their young. After weaning the young received the same treatment for 26 months. The incidence of malignant lymphatic tumours was reported as increased in all groups fed nitrite; the overall combined incidence was 31 in 573 control rats (5.4%) and 142 in 1383 treated rats (10.3%). A U.S. Governmental Interagency working group did not agree with the histopathologic diagnosis of Newberne and reported a smaller number of malignant lymphoma. They considered other lesions reported to be lymphomas by Newberne as extra-medullary haematopoiesis, plasmocytosis or histiocytic sarcomas. These tumours have no known human counterpart (Dickson, 1980).

Two months old male Sprague-Dawley rats were given water containing 0 or 2000 ppm $NaNO_2$ (equivalent to 200 mg/kgbw/day) for a period of 14 months. During this period the methaemoglobin level in test animals fluctuated from 1 - 35% as compared with 0 - 2% in the controls. The nitrite dosed rats showed decreased body and liver weights, and an increased lung weight. The lungs of nitrite dosed rats showed micro-abscesses and congestion typical of chronic pneumonitis. Further nitrite treatment caused a decrease in plasma vitamin E and an increase in reduced glutathione in red blood cells (Chow et al, 1980).

Groups of 50 male and 50 female 8 week F344 rats were given 20 ml of drinking water per day containing 0, 0.125 or 0.25% nitrite for a period of 2 years. In the female high dose group the mean body weight was decreased by more than 10 % compared to controls after 40 weeks. The survival after 100 weeks was significantly higher in the male low and high dose group. There was no significant difference in tumour incidence between treated and control rats except for high dose females which showed a significantly lower tumour incidence than control females (Maekawa et al, 1982). A level of 0.25% in drinking water was equivalent to a dose of 125 mg/kgbw/day.

Olsen et al (1984) performed a carcinogenicity study over two generations of Wistar rats. The 70 male and 140 female F0 rats were distributed into 6 groups, 3 control groups on different basal diets and 3 groups fed on a meat diet containing $NaNO_2$ at 200, 1000 or 4000 ppm of diet. Analysis of the final diets showed 2 and 2 mg/kg for the two control groups and 4, 4 and 94 mg/kg for the 3 meat groups. The presence of volatile N-nitrosocompounds up to 30 ppb N-nitrosodimethylamine was demonstrated only in meat to which 4000 mg/kg nitrite had been added. At termination after 122 weeks there was no significant difference in survival, in body weight or incidence of malignant tumours between nitrite and the control groups. However, in the total cancer incidence was increased in the 4000 ppm dose group. The highest no adverse effect level was 1000 ppm sodium nitrite in the diet, equal to a daily dose of 50 mg/kgbw when combined with a meat containing diet.

Two groups of 24 male and 24 female F344 rats received diet ad libitum, containing 0 or 2000 ppm of $NaNO_2$ and two other groups drinking water with 0 or 2000 ppm $NaNO_2$ at 20 ml/rat/day for 2 years. Occasional analyses showed that nitrite was present at levels within 5% of the nominal concentrations. There was little difference in survival between the treated and control groups. The incidence of liver neoplasms was increased in nitrite dosed females but the incidence of monocytic leukemia was decreased in nitrite treated male and female rats compared to the control rats. The test animals received daily doses of about 100 mg sodium nitrite/kgbw (Lijinsky, 1984).

6.3.4 Mutagenicity

Nitrous acid (HNO_2) is mutagenic in Salmonella typhimurium and Escherichia coli (Kaudewitz 1959, Thomas et al, 1979). At neutral pH reverse mutations occur in Salmonella typhimurium and chromosome aberrations in a culture of Chinese hamster fibroblast cell line (Ishidate et al, 1984).

Tsuda and Kato (1977) exposed newborn hamster cells to sodium nitrite (2100 mg NO_2^- l^{-1}, 4200 mg NO_2^- l^{-1}) for 24 hours and observed aneuploidy, chromosomal aberrations, and malignant cell transformation.

Negative results were found in a Host Mediated Assay using a dose of 150 mg $NaNO_2$/kgbw and Salmonella typhimurium (Couch and Friedman, 1975).

Hamsters were administered 125, 250 or 500 mg $NaNO_2$/kgbw by gavage on days 11 and 12 of pregnancy. After 24 hours cell cultures were prepared from the the foetuses. A dose-dependent increase was seen of micronuclei and 8-azaguanine and ouabaine resistant mutants in cultured cells, but there was no increase in chromosomal aberrations (Inui et al, 1979).

$NaNO_2$ (1250 mg/l) in drinking water was administered to pregnant (day 5 - 18 of gestation) and non-pregnant rats (El Nahas et al, 1984). Chromosal aberrations were seen in bone marrow of pregnant and non-pregnant adults and in liver of transplacentally exposed embryos; the magnitude of the effect was greater in embryonic liver cells than in adult bone marrow cells. This finding suggests embryonic tissue may be more susceptible in comparison with adult tissue for sodium nitrite.

6.3.5 Reproductive and foetotoxic effects

Groups of 4-6 guinea pigs containing at least 1 male per group were given drinking water, containing 0, 300, 1000, 2000, 3000, 4000, 5000 or 10000 mg/KNO_2 for 100 - 240 days. Male fertility was not impaired.

Food and water consumption and weight gain were normal except the highest dose level, at which body weight gain was decreased. Foetal losses were total with 5000 and 10000 mg/l but there was no foetal mortality at lower dose levels (< 4000 mg/l). The no-adverse effects was at least 200 mg/kg body weight per day (Sleight and Attallah, 1968).

Sinha and Sleight (1971) studied the effect of nitrite on pregnancy. Groups of 4 pregnant guinea pigs were administered subcutaneously 50, 60 or 70 mg/kg $NaNO_2$ (2% solution in water) or 50 or 60 mg/kgbw NaCl (2% solution in water). The 50 mg $NaNO_2$/kgbw and control treatments did not effect foetal mortality; 60 mg $NaNO_2$/kgbw caused abortion 1 to 4 days after dosing and 70 mg $NaNO_2$/kgbw killed all pregnant females within 60 minutes. In subsequent experiments pregnant guinea pigs were administered s.c. 60 mg $NaNO_2$ or 60 mg NaCl/kgbw. Blood samples were collected from the dams and foetuses several times after dosing. All control foetuses were alive and methaemoglobin and nitrite were absent in blood samples. In nitrite-treated dams 96% of foetuses were dead 3 or more hours after dosing. Nitrite was present in maternal (0.7 mg/l) and foetal blood (0.1 mg/l) of nitrite-treated dams until 90 minutes after treatment. Methaemoglobin content was increased until 3 hours after treatment in maternal blood (19%) and until 6 hours after treatment in foetal blood (2%). The maximum methaemoglobin level, one hour after treatment, was 67% in maternal and 22% in foetal blood.

Gruener and Shuval (1973) studied groups of 12 pregnant rats receiving drinking water containing 2000 or 3000 mg $NaNO_2$ l^{-1} from start of pregnancy until 3 weeks after parturition. A group of 7 pregnant control rats received tap water. The methaemoglobin level in control, lower and higher dose groups was 1.1, 15.5 and 24.0% respectively. The nitrite-dosed dams developed a dose-dependent anemia. Control litters contained an average of 10 foetuses; 9.5 and 8.5 foetuses were found in the lower and higher test groups. The mortality within the first 3 weeks was 6%, 30%, 53% in the control, lower and higher dose groups. Birth-weights were similar in each group, but the body weight gain of young of nitrite treated dams was decreased by 48% and 72% in lower and higher dose groups respectively. Nitrite was not transferred in appreciable amounts to the young via the milk; the blood of young did

not contain increased methaemoglobin levels while the blood of the dams contained high levels.

An oral dose of 30 mg $NaNO_2$/kgbw produced maximum blood concentration of nitrite (3.5 mg l^{-1}) after 35 minutes in a pregnant rat. The methaemoglobin level reached a maximum of 42% in the dam after 30 minutes and a maximum of 7% in the foetuses after 60 minutes, demonstrating transfer of nitrite through the placenta. In a further experiment the nitrite level increased linearly in maternal and foetal blood over a wide range of doses of nitrite (2.5 - 50 mg/kgbw). The rise in methaemoglobin level was however much steeper at nitrite doses over 15 mg/kgbw (Gruener and Shuval, 1973).

A three generation feeding study of nitrite in Sprague-Dawley rats and a two generation feeding study of nitrite in Syrian golden hamsters was performed. They were designed to explore the dose-response relation between nitrite and tumour incidence (Shank and Newberne, 1976) (see section 6.3.3).The nitrite content of the feeds were 0 or 1000 ppm (nominal). Litter size, postnatal mortality, growth rate or life span were apparently not affected.

A two generation study on Wistar rats was carried out by Olsen et al, (1984) in which $NaNO_2$ was added to diet at nominal levels of 200, 1000 or 4000 mg $NaNO_2$/kg and actual levels of 4, 4 and 94 mg/kg. The highest nitrite containing diet also contained 30 ppb N-nitrosodimethylamine. Pregnancy rate, litter size, mean pup weight and survival were similar in all test and control groups and no teratogenic effects were observed.

6.3.6 Acceptable Daily Intake (ADI) sodium nitrite

The WHO in 1974 concluded from long-term studies, that the level 'causing no toxicological effect' was somewhat below 100 mg $NaNO_2$/kgbw/day. Subsequently an ADI was set at 0.2 mg $NaNO_2$/kgbw. Applying the normal safety factor of 100 would have resulted in an ADI of 1 mg $NaNO_2$/kgbw.

In 1976 the WHO stated :

'Several recent studies on packs of cured meats inoculated with micro organisms indicate that initial nitrite concentrations of the order of 150 - 200 mg/kg are needed for the effective control of Clostridium botulinum. Further data have become available on nitrites and on the formation of nitrosamines in vivo. A new monograph was prepared. Examination of reports of a WHO task group and the IARC working group on nitrosamines (IARC Monograph No.4, 1974) have not provided sufficient evidence to revise the temporary status of the ADI. The existing specifications remain tentative'.

In 1980 the WHO did not see any reason to revise the ADI but advised repetition the studies by Newberne (1979) in pathogen-free conditions. In the original study there was a high incidence of chronic murine pneumonia which might have influenced the incidence of lymphomas. This does not appear to have been repeated.

In our opinion the highest dose level of $NaNO_2$ without adverse effects in chronic studies was about 50 mg/kgbw/day or somewhat less suggesting that the correct ADI lies between 0.2-0.5 mg $NaNO_2$/kgbw.

6.4 Toxicity, Mutagenicity and Carcinogenic Studies on Mixtures of Nitrate, Nitrite and Nitrosatable Compounds.

A wide range of toxicity, carcinogenicity and mutagenicity assays have been performed to assess the biological effects of nitrosation. In these studies (Lijinsky, 1984; Shank and Newberne, 1976; Greenblatt and Mirvish, 1972; Andrews et al, 1982) the following common features applied:

1. virtually all employed nitrite rather than nitrate with nitrosatable substances;

2. the levels of nitrite used were usually far in excess of normal human exposures;

3. in the absence of nitrite or the nitrosatable substrate no toxic effects were observed.

Although studies in which nitrite was administered in combination with a nitrosatable compound showed increases in tumour incidence (attributable to the formation of nitrosocompounds) the few studies in which nitrate was used instead of nitrite yielded no such carcinogenic effects. For example when piperazine (18.75 g/kg) was given with 12.3 g $NaNO_3$/l drinking water to mice, no increase in tumour incidence was seen (Greenblatt and Mirvish, 1972). It should be noted that the stomach of mice have a potential for nitrate reduction at that site and that they are very susceptible to the carcinogenic effects of a number of nitrosamines (Brantom, 1983).

6.5 Summary

NITRATE

- Inorganic nitrate has a low acute oral toxicity (LD_{50} > 2g/kgbw).

- In rodents, very high doses of nitrate reduce litter size, foetal weight and growth and increase the level of methaemoglobin and the relative kidney weight.

  The highest no adverse effect dose levels are :

  - $\geq$ 500 mg KNO_3/kgbw for reproduction
  - 500 mg $NaNO_3$/kgbw for growth and relative kidney weight

- Nitrate does not cause methaemoglobinemia in rats when incorporated at 5% of the diet. This is equivalent to a daily dose of 2.5g $NaNO_3$/kgbw/day.

- Application of the standard 100 fold safety factor yields an acceptable daily intake (ADI) for nitrate of 3.65 mg NO_3^- /kgbw, but vegetarians consume more than this with no adverse effect.

- High nitrate doses *in vivo* cause mutagenic effects that are difficult to evaluate due to the interfering reduction of nitrate to nitrite.

- There is no evidence that nitrate causes cancer in animals.

NITRITE

- Nitrite given to rodents in drinking water reduces reproductive rate and growth, increases methaemoglobin levels and brings about histopathological changes in the heart, lung, liver and kidney.

  The highest no effect dose level is 50 mg $NaNO_2$/kgbw.

- Application of the 100 fold safety factor yields ADI for nitrite of 0.5 mg $NaNO_2$/kgbw although the WHO recommend a value of 0.2 mg $NaNO_2$/kgbw (equivalent to 0.13 mg NO_2^-/kgbw).

- Methaemoglobinemia is the earliest detectable parameter for evaluating nitrite toxicity.

- There is no evidence that nitrite as such causes cancer in animals.

7. TOXICITY OF NITRATE AND NITRITE IN HUMAN BEINGS

7.1 Acute Toxicity

7.1.1 Nitrate Toxicity in Man

Millions of tonnes of nitrate are handled commercially each year and, despite the widespread use of nitrate, no case of acute toxicity has been reported in industry. Extensive data exists on adult humans given large doses of nitrate for medical or experimental purposes. Animal experiments are of less value for assessing the toxicity of nitrate to man because the toxic dose of nitrate depends upon competing processes, the rates of which are not necessarily the same in man and animals. For example haemoglobin regeneration, blood uptake and excretion (into urine) and recirculation (with saliva), bacterial mediated reduction of nitrate to nitrite and the various competing reactions of nitrite.

Ellen et al (1982) administered single oral doses (0.15g NH_4NO_3/kgbw) to 12 adult volunteers with no measurable haematological effect, elevation of methaemoglobin or circulating N-nitroso compounds. One developed diarrhoea after 7 hrs., and one vomited after 12 min. 12 others were administered 9.5 g $NaNO_3$ in 750 ml water intravenously over a 1 hr period. None showed any effect to the treatment.

Ammonium and sodium nitrate have been used in 10 g/day doses for 4-5 days as diuretic; NH_4NO_3 was the more effective. The nitrate was rapidly excreted in the urine, 75% during the treatment and another 15% during the next 2-3 days. (Keith et al, 1929, 1930). Three patients on such treatment were reported to have developed cyanosis due to methaemoglobinaemia; one had undergone surgery for gastric ulcer previously and this may have reduced his ability to absorb nitrate, increasing the possibility of bacterial reduction. The other two patients had marked constipation and this may have affected the nitrate absorption and metabolism (Eusterman and Keith, 1929). Bruijns (1982) described the use of 2-9g NH_4NO_3/day to prevent renal phosphatic calculi. 268 individuals were treated for 3 weeks to 2 years; methaemoglobinaemia was reported in ony two cases, of which one

was attributed to methaemoglobin reductase deficiency. The other was not further studied.

A reported case of fatal nitrate poisoning has been described (Amundsen, 1947). 15 soldiers became ill approximately 6 hours after eating yellow turnip "salted" by mistake with sodium nitrate; 13 died of methaemoglobinaemia. It was estimated that consumption was about 15 g $NaNO_3$ for those that died, and 5 g for those that survived. The author suggests that the composition of the meal favoured slow uptake and hence increased reduction. This concept has some experimental support (Selenka, 1983; Wise et al, 1982).

7.1.2 Nitrite Toxicity in Man

The toxic effect of nitrite is well known and documented; nitrite causes depression of blood pressure and production of methaemoglobin.

Transformation of (ferrous)oxyhaemoglobin to (ferric)methaemoglobin is chemically complex and is caused also by other substances. Aniline and 4-dimethylaminophenol are among many compounds that catalyze autoxidation of oxyhaemoglobin to methaemoglobin. Nitrite oxidation of oxyhaemoglobin to methaemoglobin is generally reported to follow the equation

$4\ HbO_2 + 4NO_2^- + 4H^+ \rightarrow 4\ MHb + 4\ NO_3^- + O_2 + 2H_2O$ (Kosaka et al, 1979)

However, this molecular relationship derives from in vitro studies and is only valid when nitrite is present in considerable excess ($(NO_2^- / HbO_2 > 8)$) (Doyle et al, 1985). The reaction is complex and probably involves the intermediate formation of nitrogen dioxide and peroxide (Watanabe et al, 1981; Doyle et al, 1985; Kosaka and Uozumi, 1986; Spagnuolo et al, 1987). Stoichiometric measurements have been used to calculate toxic doses of nitrite (Winton et al, 1970) but the complexity of the reactions makes this approach dubious.

The reaction in vitro shows a lag phase followed by autocatalysis and is generally completed in 5-10 minutes. (Smith and Beutler, 1966; Tarburton and Melcalf, 1986; Kosaka and Uozumi, 1986). In vivo the reaction is markedly slower. Maximum methaemoglobin levels were

reached in about 80 min following subcutaneous injection of $NaNO_2$ in dogs, and in about 120 min wher the nitrite was fed in capsule (Jensen and Anderson, 1941).

Sodium nitrite has been used occasionally for suicidal purposes. The lethal dose seems to vary between 2-9 g $NaNO_2$ (WHO 1985), 4 g being a typical value for adults (de Beer et al, 1975). The basis for this figure was the poisoning which occurred when soup was salted by mistake with curing salt containing nitrite in 1946 in Leipzig, Germany. Of 71 persons affected, 37 required hospitalization and 7 died (Schulze and Scheibe, 1948). Other similar cases are in accord with this incident (Schmidt et al, 1949).

Kiese and Weger (1969) reported experiments in which human volunteers were given $NaNO_2$ intravenously. A dose of 4 mg $NaNO_2$/kgbw (6 experiments) produced a maximum methaemoglobin level of 7% after 30 min. A dose of 12 mg/kg bw (1 experiment) gave levels of 25% after 30 min, and 30% after 60 min. If methaemoglobin levels are proportional to the dose, this experiment indicates that the potential lethal dose for adults by injection (60% methaemoglobin level) should require about 24-34 mg $NaNO_2$/kgbw, or about 1.4 - 2.0 g for an adult of 60kg.

In man the first symptoms of oral nitrite poisoning develop after 15-45 minutes. (Schmidt et al, 1949; Standefer et al, 1979; Aquanno et al, 1981).

7.2 Methaemoglobinaemia

7.2.1 Causes, Symptoms and Diagnosis

The principle human health concern of nitrate intake is infantile methaemoglobinaemia as a consequence of nitrite formed by microbial reduction of nitrate. Methaemoglobin does not carry oxygen and excess levels lead to cyanosis and tissue anoxia.

Concentrations above 10% can be diagnosed clinically by cyanosis but below this level spectroscopic analysis of blood is required; blood analysis is essential to confirm diagnosis. During analysis

haemoglobin is converted to cyanomethaemoglobin with a characteristic absorption band between 600 and 660 nm. Methaemoglobin is usually expressed as percent of total haemoglobin present (Hegesh et al, 1970); most authors use this terminology and we have adopted this usage rather than absolute g % methaemoglobin. Methaemoglobin is not measured routinely in clinical practice and limited information is available for either infants or adults to define normal and abnormal concentrations. The precision of the analysis is not great, as shown in the spread of data between authors for normal background values.

Normal methaemoglobin levels given in the literature are listed (Table 7). They cover a wide range but levels of 0.5-2.0% appear to represent the normal range; newborn have higher levels than older infants. WHO (1984b) refer to 1-2% methaemoglobin as normal. At these levels there are no demonstrable clinical effects (Jaffe, 1981; Craun et al, 1981). When the level rises to an ill-defined level (about 10%), clinical signs are evident and the condition is termed methaemoglobinaemia. Death can result when between 45 to 65% haemoglobin has been converted (Sattelmacher, 1962). Neonates have an incompletely developed system for methaemoglobin reduction and are thus at special risk. Individuals with methaemoglobinaemia of >60% have been reported to recover (Thal et al, 1961). Skrivan (1971) claims that pregnant women have markedly elevated methaemoglobin levels (up to 10%), but confirmatory data are lacking.

Methaemoglobinaemia is a very rare disorder which has a number of causes including inherited metabolic deficiencies and accidental exposure to certain chemicals (Kiese, 1974; Jaffe, 1981). The conversion of oxyhaemoglobin to methaemoglobin can be catalysed by substances such as sulphonamides and phenacetin (Sattelmacher, 1962) as well as amylnitrite and glycerol trinitrate (Tarburton and Metcalf, 1986). The last two have been utilized to induce rapid methaemoglobin formation in the treatment of cyanide poisoning (cyanide preferentially binds to methaemoglobin iron). Such methaemoglobinaemia is now well recognised and is seldom reported in the medical literature.

Among the chemicals that can oxidize haemoglobin to methaemoglobin is nitrite. The majority of cases of methaemoglobinaemia described since 1945 are associated with nitrite in foods (Orgeron et al, 1957 ; Lee, 1970) or with high nitrate concentrations in well-water used for making infant milk formulations.

Symptoms of the disease result from oxygen deprivation (dyspnoea, stupor, coma and death due to anoxia). The condition is readily treatable by intravenous injection of methylene blue which produces a rapid response, normally with no significant sequellae. For infants no short term impairment of health has been seen after recovery from an acute attack (Comly, 1945) but no long time follow up of cases has been reported. In view of the lack of problems in heriditary methaemoglobinaemia, in which 10-20% methaemoglobin is a permanent condition, no chronic effects are to be expected (Jaffe, 1981).

Salicylates are of interest as these inhibit the enzyme diaphorase which slowly reforms haemoglobin from methaemoglobin (Worathumrong and Grimes, 1972). Thus there is a possibility that the use of salicylates to treat the fever associated with infantile enteritis might aggravate a latent or manifest methaemoglobinaemia. This is one of many possible confounding factors that complicates the interpretation of such cases.

7.2.2 History of Well-water Methaemoglobinaemia

7.2.2.1 Background

In 1945 Comly described well-water methaemoglobinaemia in two children 33 and 27 days old. The association between nitrate in water and the disease stems from this paper. Poor water quality was linked by circumstantial evidence to cyanosis and the failure of infants to thrive. He noted that the infants were suffering from gastro-intestinal infections and were given polluted water containing 388 and 619 mg NO_3^- l^{-1}, and 1.3 and 0.4 mg NO_2^- l^{-1} respectively as their food diluent. Gross bacterial contamination existed with 240 coliforms/100cc; the water was drawn from shallow wells.

Comly mentioned anecdotal evidence from elsewhere in the United States of methaemoglobinaemia associated with well-water nitrate levels of 283-620 mg l^{-1}. He recommended that to protect the young infant well-water nitrate levels should be ideally below 45 mg l^{-1} and not more than 90 mg l^{-1}. The advice has been widely accepted. Bailey (1966) reviewed 40 American cases and found that girls were more susceptible than boys, but this has not be confirmed elsewhere. Since 1945 about 3000 other cases have been reported (WHO, 1985), some with fatal outcome.

As the early authors use the name "well-water methaemoglobinaemia" to describe this condition we have likewise followed this terminology.

The enigmatic character of the condition was well described by Donahoe (1949):

> "It is difficult to explain why only an occasional infant develops cyanosis, why the nitrate content of the water (associated with the cases) varies so greatly and why it is not always the water with the highest nitrate concentration which causes cyanosis in the infant".

Acute methaemoglobinaemia might have a number of causes other than exposure to nitrate and there is thus a degree of uncertainty which cases should be accepted as associated with nitrate. Furthermore cases were usually evaluated without an awareness that methaemoglobinaemia can be a complication of enteritis and its treatment, independent of nitrate intake. The literature should therefore be reviewed with these points in mind.

7.2.2.2 Geographic Distribution

In his world-wide survey Sattelmacher (1962) reported the number of published cases in different countries :

Europe		Africa	2
Czekoslovakia	319	America	
Germany	255	Minnesota	146
Austria	22	Illinois	75
Belgium	20	Iowa	69
France	17	Nebraska	26
Hungary	8	Others	57
Others	12	Canada	32
		TOTAL	1060

Well-water methaemoglobinaemia has always been a rare disease even in those areas where it occurred most frequently. Of recent years Hungary appears to be the sole State where the condition continues regularly. Steiner and Buryevacz (1981) reported that in the Hungarian county of Bargana 234 cases occurred in 80,370 live births (0.3%) in 1968-79. Most communities in this area (59%) were supplied with hygienically unsatisfactory water, but it is not known to what extent such water was used for infant feed preparation. Deak (1985) stated that 1353 cases (including 21 deaths) occurred in Hungary between 1976 and 1982 and all were connected with privately dug wells. In most cases the water nitrate concentration was above 100 mg^{-1} but in 7% it was 40 - 100 mg^{-1}. Few details are available about the case distribution within this concentration range, or the age, health status, any other medical details, or information about water quality apart from nitrate concentration. Thus it is not possible to evaluate further this unusual Hungarian epidemic, but compared with elsewhere it appears to be a special case.

Simon et al (1964) surveyed all cases of "alimentary methaemoglobinaemia" in child disease departments in German hospitals using a questionnaire. 745 cases were recorded. Geographical distribution was uneven with most cases recorded in East Germany (86%). Most patients were younger than 3 months of age; methaemoglobinaemia was associated with enteritis in 53% of cases. Amendt and Reddermann (1969) reported that the number of

cases in Greifswald, Germany, fell markedly between 1959 and 1967, whilst Borneff (1986) related that no cases had been seen in the Mainz area for the past 25 years, despite a nitrate content up to 400 mg NO_3^- l^{-1} in well-waters in the area.

In the UK the last reported death was in 1950 and the last confirmed case in 1972; of the 14 suspected cases reported over the last 35 years all used well water with nitrate concentrations greater than 100 mg l^{-1} (Acheson, 1985).

In parts of Jutland, Denmark, the nitrate concentration in ground water may rise to above 50 mg l^{-1} but the last case of methaemoglobinaemia occurred in 1976, associated with a polluted well containing 200 mg NO_3^- l^{-1}. No other cases were found between 1977-82 (Hye Knudsen, 1985).

A minor epidemic (9 mild cases) occurred in Spain in 1980 (Farre Sostres et al, 1982), subsequently discussed in Section 7.2.5.1. Cases occur occasionally in Poland (Swiatkowska and Gockoswka, 1981) but the incidence is not known.

The disease has almost disappeared from Czechoslovakia (Fadrus and Kvet, 1982). Our efforts to identify other European cases have failed. Although not a notifiable disease, Western European Governments' appear to document the extent of the condition and had deaths occurred they would have been registered. This suggests that well-water methaemoglobinaemia is very rare in Western Europe.

A similar decline has been noted in the USA. Choquette (1980) reported methaemoglobinaemia following use of well-water containing 1200 mg NO_3 l^{-1} and stated :

> "less severe cases have been reported following consumption of water in the ranges above 200 mg NO_3^- l^{-1}."

Of recent times reported cases include one in South Dakota where a 6 week old infant was given feed made with well-water with 545 mg

Assuming a normal liquid intake for infants of 150 ml/kgbw/day (Hull and Johnstone, 1987) these doses are equivalent to nitrate concentrations in water of 330 and 660 mg l^{-1}; the actual concentrations could be somewhat higher or lower since individual intakes do vary. Feeding 100 mg NO_3^-/kgbw/day to two infants who had recently recovered from well-water methaemoglobinaemia gave some cyanosis and a methaemoglobin level of 11% in one of the infants.

Toussaint and Selenka (1970) gave 2 groups of 14 and 20 infants feed made with 150 NO_3^-/l^{-1} for 10 days. The maximum methaemoglobin level was 3%. In addition they examined the effect of adding 25 or 35 mg NO_2^- l^{-1} to the feed; at the lower concentration the methaemoglobin levels were hardly affected during 10 days feeding, whilst at 35 mg NO_2^- l^{-1} it increased to 3-7%.

Winton et al (1970), in a widely quoted paper, mention that an infant given 15.5 mg NO_3^-/kgbw/day (approximately 100 mg NO_3^- l^{-1}) had a methaemoglobin level of 5.3 %. On change to " low nitrate water" the level fell to "within the normal range" which in this study was 0-2.9%.

Gruener and Toplitz (1975) and Shuval and Gruener (1977) studied 104 infants aged 1 week to 10 months old hospitalized mainly with gastro-intestinal conditions. The hospital normally used water with a high nitrate concentration. On the first day the infants were given a formula feed made with water containing 15 mg NO_3^- l^{-1} (no information is given regarding the earlier feeding regime). This was followed by feeding for 3 days a formula feed made with water containing 108 mg NO_3^- l^{-1} , and then on a feed made from water at the lower nitrate concentration. There was no separate control group and no clinical details are given. Following the change from low to high nitrate, the mean blood methaemoglobin rose from 0.89 to 1.3%. Within the group (n=93) 59% of infants showed an increase, yet 37% showed a decrease. Three of the infants recorded methaemoglobin levels of 7, 14 and 17% (surprisingly without cyanosis), but no further details were recorded. The results were taken to indicate that adaptation takes place to changes in water nitrate concentration.

7.2.3.2 Field Investigations with Infants

Shuval and Gruener (1977) reported that 22 infants fed powdered milk made with tap water containing an average 87 mg NO_3^- l^{-1} had 1.4% methaemoglobin, compared with 0.7% in the control group using water with an average concentration of 32.5 mg NO_3^- l^{-1}. The difference was less for infants on feed combinations of both powdered milk and liquid milk.

Würkert (1978) reporting data from 1971 found that 68 infants using water with 0-30 mg NO_3^- l^{-1} had average methaemoglobin levels of 1.80%, max. 7.07%, while 24 infants fed water with 31-100 mg NO_3^- l^{-1} and 4 given water with more than 100 mg NO_3^- l^{-1} averaged 3.49%, max. 14.1%. These ranges are markedly outside those of other authors.

Borneff (1980, 1986) reporting from the same area in Germany found a tendency towards higher methaemoglobin concentrations when the water contained 51-90 mg NO_3^- l^{-1} compared to 0-50 mg NO_3^{-1} l^{-1} but stated that it is somewhat doubtful if the variations found were beyond the normal range. Fernicola and Azevado (1981) found that 92 Brazilian children (average age 15 mo) who drank water with less than 45 mg NO_3^- l^{-1} had an average methaemoglobin level of 0.56%, whilst 24 children that used water above this limit (no further details) had 0.76%. Similar results were described in Poland by Pilawska (1976); 32 infants were fed mixtures made with water containing 16 $\pm$3 mg NO_3^- l^{-1} had methaemoglobin levels of 0.29 $\pm$0.05%, whilst 88 infants fed with feed made from water with 212 $\pm$ 15 mg NO_3^- l^{-1} had 0.75 $\pm$ 0.08%. All these results seem to be in the normal physiological range for methaemoglobin levels. It is doubtful if the analytical precision in the methaemoglobin analysis is so reliable that these small differences are really meaningful.

Gelperin et al (1971) studied two groups of 12 and 11 newborn in Dauville, Illinois. Water with 45 mg NO_3^- l^{-1} produced average methaemoglobin levels of 1.91% (max. 5.87%) whilst 37 mg NO_3^- l^{-1} produced levels of 0.09% (max. 1.26%). Elevated levels were also found in a few of the mothers. The results are so much outside the

range found by others, that they can not be accepted without confirmation.

Super, Heese et al (1981) studied 0-12 months old infants in rural Namibia. The families were visited by a health team, feeding and health condition noted, appropriate treatment given when indicated, and a blood sample analysed in a mobile laboratory. In the paper the analytical values are ambigously expressed in the report. They should be read as NO_3-N (Heese, 1987). The authors found that infants living in areas with water nitrate levels above 90 mg NO_3^- l^{-1} tended more often to have elevated methaemoglobin levels (above 2-3%) than those using water with less than 90 mg NO_3^- l^{-1} (20 mg NO_3-N/l, not 4.5 mg/l as stated in the WHO report, (1985a).

Water NO_3^- mg/l	Numbers (and Percentage) of infants with methaemoglobin levels of		
	≤ 2%	2.1 - 3.0%	> 3.0%
≤ 90	228 (78%)	27 (9%)	38 (13%)
> 90	107 (55%)	22 (11%)	64 (33%)

There was no difference between the age groups and no correlation was found between methaemoglobin levels and diarrhoea and/or respiratory infections. No conclusions can be drawn about the correlation of methaemoglobin levels with nitrate intake in the concentration range below 90 mg NO_3^- l^{-1} that is of primary interest here. However, even the control group had methaemoglobin levels higher than normal ; no explanation for this is available.

7.2.3.3 Field Investigations with Older Children

Diskalenko (1968) measured the methaemoglobin levels of children from rural areas in the USSR (up to 9 years old) and the nitrate concentration in their drinking water. In 2 of the 3 groups studied

there were exceptionally high water nitrate concentrations and elevated methaemoglobin levels :

No. of Children	Water Nitrate (mg $NO_3^- l^{-1}$)	Methaemoglobin range (%)
234	<45	1.4 - 1.8
126	808	2.1 - 3.3
157	921	3.1 - 7.5

Craun et al (1981) studied 102 children, aged 1-8 years, and did not find methaemoglobin levels above the norm with drinking water nitrate concentrations of 97-492 mg l^{-1}. Takacs et al (1970) found that a group of 169 Hungarian children (1-7 years old) living in communities with hygienically unsatisfactory well water in which the nitrate concentration lay between 134-500 mg l^{-1} had an average methaemoglobin level of 1.63% in the summer and 0.83% in the winter. A control group (149 children) supplied with water containing less than 10 mg $NO_3^- l^{-1}$ had 0.6% in the summer and 0.5% in the winter.

7.2.4 Methaemoglobinaemia and Water Nitrate Concentration

7.2.4.1 Well-water Methaemoglobinaemia associated with the use of water above 100 mg $NO_3^- l^{-1}$

In a review of methaemoglobinaemia cases in the USA the majority of the 214 cases for which water data were available, were associated with intake concentrations greater than 220 mg $NO_3^- l^{-1}$ (Walton, 1951). Only 5 cases were associated with water containing 50-90 mg $NO_3^- l^{-1}$. No medical details were given, except that "methaemoglobinaemia is commonly accompanied by diarrhoea or other gastrointestinal disturbances". These cases can therefore not be evaluated.

Sattelmacher (1962) reviewed the 467 published cases of methaemoglobinaemia for which the water nitrate concentration was available. Data from the review by Walton (1951) were included. Sattelmacher reports:

	Nitrate concentration in water used for feed preparation (mg NO_3^- l^{-1})			
Methaemoglobinaemia cases	0-40	41-80	81-100	>100
Number	14	16	19	418

This (and the similar earlier observation by Walton, 1951) form the factual basis for the original water quality criterion that potable water should not contain more than 100 mg NO_3^- l^{-1}. This point of view is not presently in dispute.

7.2.4.2 Well-water Methaemoglobinaemia Associated with the Use of Water with less than 100 mg NO_3^- l^{-1}

We have used the Sattelmacher (1962) review to identify cases associated with water nitrate concentrations below 100 mg l^{-1}, see Table 8 which also incorporates the cases published after 1961. Two of the Sattelmacher's papers have been omitted either because of non-accessibility (Vetter, 1959) or misclassification of the nitrate concentration (Boisson et al, 1956). Most papers provide inadequate medical and/or water supply details. In two, the water analysis figures are ambiguous and may be expressed in terms of NO_3^- or NO_3-N; it is assumed that NO_3^- was intended.

Amendt and Reddermann (1969) reported that 6 cases out of 77 from Greifswald, Germany, received drinking water <100 mg NO_3^- l^{-1}; the lowest nitrate concentration was 37 mg l^{-1}. Simon et al (1964) mentions some of the above cases, together with others; they found

16.2% of cases were associated with water concentrations less than 100 mg NO_3^- l^{-1}. Deak (1985) stated that 7% of all Hungarian cases, observed in 1982 (i.e. 97) were associated with nitrate levels less than 100 mg l^{-1}; no details are provided excepting that all cases were associated with private wells.

Table 8 clearly shows (where details are provided) that the most noticeable feature is the unsatisfactory hygienic state of the well-water. An elevated nitrate concentration in water has long been used as an indicator of pollution (e.g. bacteriological). The table suggests that factors other than nitrate may have caused the methaemoglobinaemia at these low nitrate concentrations.

No references connecting methaemoglobinaemia with the consumption of piped water supplies containing nitrate levels less than 100 mg l^{-1} have been located with the exception of four papers by Betke and Kleihauer (1957), Vigil et al (1965), Verger et al (1966), and Aussannaire et al (1968) which are commented upon in sections 7.2.5.1 and 7.2.5.3. The circumstances in all these 4 cases suggest that factors other than nitrate caused the disease.

7.2.5 Methaemoglobinaemia and Enteritis

7.2.5.1 Methaemoglobinaemia, the Intestinal Microflora and Intestinal Infections.

The importance of factors other than nitrate for the aetiology of methaemoglobinaemia is apparent from case descriptions. Horn (1958) mentions cases where one infant has developed methaemoglobinaemia, while other infants (including in one case a twin) receiving water from the same nitrate rich wells showed no signs

Once an infant has developed methaemoglobinaemia the risk for re-occurrence seems greatly increased (Comly, 1945; Donahoe, 1949). A possible explanation is that an intestinal flora favouring nitrate reduction is present in such infants (Cornblatt and Hartman, 1948; Horn, 1958). The flora of the infant gut during the

first weeks after birth is determined by exposure to micro-organisms and diet (Borrielo and Stephens, 1984). The high buffering capacity and lower lactose content of cows' milk preparations favours the growth of Enterobacteriaceae rather than the Lactobacillia which predominate in the stools of breast-fed babies (Bullen & Willis, 1971). Nitrate reduction is unusual among lacto-bacilli (Bergey, 1974), and an intestinal flora dominated by lactobacilli may have a lower capacity for nitrate reduction than one where enterobacters are a major proportion of the bacterial flora.

It is a general experience, dating from the first described cases (Comly, 1945), that infant methaemoglobinaemia is often associated with gastro-intestinal upsets. This is also stressed by Sattelmacher (1962) in his extensive review.

Older medical literature (e.g. Talma, 1902; Stockvis, 1902; Berg v.d., 1905,1906; Gibson and Douglas, 1906 and Lichtenbelt, 1923) describes cases of methaemoglobinaemia associated with gastrointestinal infections in adults. Unfortunately the relevant circumstances of nitrate intake of the patients were unknown or poorly documented, and the probable causes can not now be properly assessed.

Ceck et al (1964), Worm (1964) and Zanesco (1964) gave the first definite descriptions of methaemoglobinaemia as a complication of gastro-enteritis in infants with low or negligible nitrate intake. In Worm's case the water consumed contained 1 mg NO_3^- l^{-1} and two of Zanesco's 7 cases were breast-fed premature infants (human milk contains less than 5 mg NO_3^- l^{-1}). Ceck et al (1964) described well-water methaemoglobinaemia in a 3 week old infant with diarrhoea, the water contained 139 mg NO_3^- l^{-1}. During subsequent hospitalization without nitrate exposure further incidents of methaemoglobinaemia and diarrhoea occurred, after 8, 15 and 27 days. These cases illustrate the important role of enteritis in the development of methaemoglobinaemia in infants, a feature seen by many other authors. The common features are diarrhoea (usually with green stools), metabolic acidosis, and cyanosis.

There are 75 or more further cases of methaemoglobinaemia due to enteritis recorded in the literature, cf. Caille and Herouin (1969), 3 cases; Barna et al (1974), 9 cases; Yano et al (1982), 11 cases; Bricker et al (1983), 15 cases; Hanukouglu et al (1983), 9 cases; Seeler (1983), 5 cases; Danish (1983), 5 cases; Blanc et al (1983), 4 cases; Maloney et al (1983), 1 case; May (1985), 1 case; Mailfert et al (1986), 1 case; Lanir et al (1986), 11 cases. Shearer et al (1972), and Shuval and Gruener (1972) noted elevated but subclinical methaemoglobinaemia associated with diarrhoea. Kohne et al (1974) described two cases in which enteritis was due to cow's milk intolerance.

Methaemoglobinaemia with infant enteritis has occurred as epidemic episodes. Fandré et al (1962) treated 54 infants with enteritis in Reims (France), of which 19 developed methaemoglobinemia. In six carefully studied cases the methaemoglobin concentration varied from 15 to 59%. The infants were fed with powdered baby food in water of low nitrate content ('Evian' mineral water). The author concluded that the methaemoglobinemia originated from endogenous nitrite produced by entero-pathogenic coliform bacteria.

A gastro-enteritis epidemic affected 50 infants in Spain, and produced cyanosis and methaemoglobinaemia in some cases (Farre Sostres et al, 1982). The community water contained 76 mg NO_3 l^{-1}; clinical data are summarised in table 9. The Spanish cases are similar to those described by Yano et al (1982) and others and it seems highly likely that the methaemoglobinaemia was a complication of gastero-intestinal infection rather than nitrate intake. The findings of Sostres et al (1982) are often given as evidence for nitrate causing methaemoglobinaemia. In our opinion the cases show many common features with those investigated by Fandré et al (1962) in which infection was the cause.

Even so, enteritis induced methaemoglobinaemia is rare. Berg and Rappe (1976) stated that 21 Swedish infants hospitalized with diarrhoea had methaemoglobin levels below 2% and as Barna et al (1974) found that few enteritis patients developed elevated methaemoglobin levels.

The origin and causes of this type of methaemoglobinaemia have only been sparingly researched. Hegesh and Shiloah (1982) have shown that methaemoglobinaemia can be caused by endogenously synthesised nitrate and consider it independent of nitrate intake. Lanir et al (1986) suggested that foetal haemoglobin may easily be converted to methaemoglobin at reduced pH and that this is why the condition is found mainly in young infants and associated with acidosis. Further work in this field is desirable.

The occurrence of methaemoglobinaemia in infantile gastroenteritis means that methaemoglobinaemia ascribed to high nitrate levels in infant feed water may in fact have been the result of infection.

For example Betke and Kleihauer (1957) described a 10 day old infant, with enteritis that was spoon fed piped public water containing 33 to 55 mg NO_3^- l^{-1}, as it had difficulties with drinking due to an ear and throat infection. Vigil et al (1965) described a 4 week old infant that developed diarrhoea and then methaemoglobinaemia. Its feed was made with "polluted" municipal water containing 73 mg NO_3^- l^{-1}. The infant was treated in hospital but relapsed and further treatment for methaemoglobinaemia was required. In both cases the authors ascribed the condition to nitrate in the water but clearly both could have been ascribed to gastro-enteritis.

7.2.5.2 Methaemoglobinaemia, Infant Nutrition and Bacterial Contamination

Nitrate concentration of liquid milk is generally low (Luf and Brandl, 1986). Simon et al (1964) found 0-0.5 mg NO_3^- l^{-1} for cow's milk, and between 0.5-3.4 mg l^{-1} for mothers milk.

Many papers reporting cases of methaemoglobinaemia provide little or no information on the reconstituted milk used in the infant feeds.

In the review of German cases by Simon et al (1964), the type of feed was known in 304 cases; 116 cases received powdered milk, 138 diluted cows milk, 26 a combination of mothers milk and milk

preparation and 24 special diet (unspecified) for diarrhoea. Only 18 of the cases had acidified milk feeds. Knotek and Schmidt (1964) noted that none of the 115 cases of methaemoglobinaemia in Czechoslovakia had been given feed made from a commercial dried milk based on buttermilk (which is slightly acidic). The methaemoglobinaemia positive cases had been given feed made from 3 other commercial brands or normal dried milk. All the latter products had spores of B. subtilis with nitrate reducing potential. Use of buttermilk or acidified milk reduces the propensity for nitrate reduction in the feeds.

Bacteria present in milk may play a role in the development of methaemoglobinaemia. Popp (1960, 1976) mentions experiments which showed that nitrite was generated from powder milk preparations when diluted with water. He stated that the nitrite formation was generally independent of the amount of nitrate present in the water. No nitrite was produced when the diluted milk preparation was boiled but it was produced when boiled water was used for preparing the mixture without subsequent sterilization. He concluded that an important factor in the genesis of methaemoglobinemia could be the use of milk powder heavily contaminated with nitrate reducing bacteria.

Simon et al (1962) investigated the formation of nitrite in feeds made from sterile water containing 40-60 mg NO_3^- l^{-1}, and powdered milk with and without lactic and citric acids, at 22° or 37°C after inoculation with bacteria. Extensive nitrite formation (20-30 mg/l) was found after 12 hrs. E. coli was especially efficient and reduced nitrate at 22°C while Aerobacter aerogenes and Staphylococcus aureus required the higher temperature for extensive reduction. Acidification of the milk suppressed the formation of nitrite. Cornblatt and Hartman (1948) stated that methaemoglobinaemia could be prevented by adding lactic acid to milk formulae when the water used was contaminated with nitrate. In contrast, Toussaint and Selenka (1970) found that nitrite formation in a milk formulation with (apparently) added coliform bacteria and water containing 150 mg NO_3^- l^{-1} was slow, giving only 3 mg NO_2^- l^{-1} after 20 hours. Lactobocilli do not normally form nitrite, and the

bacterial processes taking place in contaminated milk preparations may be multiple and difficult to reproduce.

Bacterial contamination of the water is a regular feature of well-water methaemoglobinaemia and the milk provides an alternative source of bacteria. At the present time feed preparations based on cows' milk are used for infant feeding on a large scale with few problems, no doubt aided by improved hygienic conditions and treatments to reduce bacterial contamination. How much the milk component of infant feeds contributed to methaemoglobinaemia problems in earlier times is unanswered but pertinent to the aetiology of well-water methaemoglobinaemia.

7.2.5.3 Control of Infant Diarrhoea

Infant methaemoglobinaemia resulting from the consumption of nitrate in vegetables is known (Lee, 1970 ; Ritter and Schulze, 1971 ; Keating et al, 1973, Thunnissen and Zwart, 1985) generally due to bacterial reduction of contaminated stored feed. Normally vegetable feeds are not given to infants less than 3 months old. Nevertheless infantile diarrhoea has been treated with carrot puree. It has been suggested that the indigestible fibre increases the viscosity of the intestinal content, reduces intestinal mobility and thus reduces the water and electrolyte loss that make infant diarrhoea a potentially dangerous condition. This treatment has been advocated for home use. (Pernoud, 1972). Carrots may contain 1g NO_3^- kg^{-1} (table 2) and stored carrot soup may become rich in nitrite; it has been shown that use of carrot soup for diarrhoea treatment may indeed cause infantile methaemoglobinaemia (L'Hirondel et al, 1971; Seguin et al, 1977; Kovacs et al, 1981; Kralinkowa et al, 1982; Blanc et al, 1983). When commercial carrot puree with nitrate content controlled at 50-80 mg NO_3/kg was used for preparing the diarrhoea diet, no increased methaemoglobin levels resulted (Lindquist and Søderhjelm, 1975).

Verger et al (1966) described methaemoglobinaemia in 2 infants given carrot soup made from municipal water (30 mg NO_3^- l^{-1}); the carrots were not analysed. Similarly Aussannaire (1968) describes

11 cases in which infants were given home-made carrot soup to prevent diarrhoea prior to developing methaeblobinaemia ; in this case the municipal water contained 70 mg NO_3^- l^{-1}.

It is impossible now to estimate how much the practice of treating infantile diarrhoea with home made carrot puree contributed to methaemoglobinaemia observed earlier and classified as well-water methaemoglobinaemia. However, Simon et al (1964) found that of 304 German cases of infant methaemoglobinaemia, 24 were on a special (unspecified) diet for diarrhoea. This and the other cases mentioned above suggest that the contribution of carrot puree may not have been negligible.

7.2.6 Methaemoglobinaemia: Evaluation.

Even in 1945-65 well-water methaemoglobinaemia was a rare disease and is now virtually non-existent in Western Europe. The evidence indicates that a number of factors have to be present for the condition to develop (WHO, 1985a). Enteritis is perhaps the most important especially when the water is of poor bacteriological quality drawn from private wells. There are many instances where infants have received water containing nitrate at concentrations greater than 100 mg l^{-1} without showing adverse effects. No cases have been found in the literature in which infantile methaemoglobinaemia was associated with concentrations of water nitrate below 100 mg l^{-1} unless the water was bacterially contaminated or unless the infants were already suffering from enteritis. These factors combined suggest that the bacteriological, rather than the nitrate, status of the water is a key aetiological factor in infantile methaemoglobinaemia.

Evidence indicates that use of water within the range 50-100 mg NO_3^- l^{-1} is associated with infantile methaemoglobin levels within the normal physiological range. It remains a possibility that the use of such water for infant feeding may produce levels at the high end of this normal range.

The WHO (1984a) guidelines on drinking water quality state, with reference to water with a nitrate content between 45 and 90 mg NO_3^- l^{-1} (i.e. 10-20 mg NO_3-N/l) that :

> "although the clinical manifestations of infantile methaemoglobinaemia may not be apparent at these levels, undesirable increase in methaemoglobin in blood do occur. For this reason a guideline value of 10 mg of nitrate-N per litre is recommended".

There is no evidence in the literature to suggest that the observed minor increases within the normal physiological range are harmful. In our opinion the reduction in the maximum acceptable level from 22.6 mg NO_3-N/l to 10 mg NO_3-N/l in water supplies with a satisfactory bacteriological standard is not justified by the evidence.

7.3 Birth defects, Cardiovascular and Thyroid Effects.

Nitrate was reported as inducing a high incidence of foetal abnormalities in the Mount Gambier region of Australia (Scragg et al, 1982); a subsequent report by the same group failed to confirm the observations (Dorsch et al, 1984).

The hypothesis that nitrate may have an effect upon the cardiovascular system or the thyroid have received some study. The topic was reviewed by WHO (1985a) whose conclusion, with which we concur, was :

> " Studies relating congenital malformations and cardiovascular effects to nitrate levels in drinking-water have not produced consistent results. Some animal studies indicate that chronic exposure to high levels of nitrates can reduce the intrathyroid iodine pool and thus render the gland more sensitive to goitrogens. However, whether or not exposure to nitrate is an etiological factor in human goitre remains to be determined."

7.4 Summary

- Sodium and ammonium nitrate show low toxicity to man and have been used for the treatment of phosphatic kidney stones at rate up to 9 g/day without adverse effect.

- The lethal oral dose of sodium nitrite for adults is about 4 g.

- The normal range for methaemoglobin is 0.5-2%.

- When the conversion to methaemoglogin exceeds 10% in the blood the condition is termed methaemoglobinaemia.

- Infantile methaemoglobinaemia has been associated with bacterial contamination of water and food, bacterial infections, nitrite contamination of infant food, and high nitrate content of drinking water.

- The acute infantile methaemoglobinaemia associated with the use of water for preparation of milk for bottle feeding is associated only with the use of well-water and was thus termed well-water methaemoglobinaemia.

- Well-water methaemoglobinaemia is a rare condition, the incidence of which has decreased in Western Europe in the past 20 years and is now virtually non-existent.

- Nitrate does not cause methaemoglobinaemia; it has to be reduced to nitrite to induce the condition.

- Methaemoglobinaemia may occur with a low nitrate intake and this is thought to be associated with enhanced endogenous nitrate synthesis due to enteritis.

- The great majority of cases of well-water methaemoglobinaemia occurred when nitrate levels exceeded 100 mg l^{-1}.

- In those cases where methaemoglobinaemia was associated with water containing less than 100 mg NO_3^- l^{-1} the water was of poor hygienic quality, and the infants showed signs of gastroenteritis.

- There is no evidence that methaemoglobinaemia is caused by bacterially sound water supplies at concentrations up to 100 mg NO_3^- l^{-1}.

8. NITRATE AND CARCINOGENESIS IN MAN

8.1 Mutagenicity

The mutagenicity of nitrate and nitrite in human leucocytes has been examined by measurement of unscheduled DNA synthesis (UDS; a response to DNA damage) following ingestion of meals containing different levels of nitrate (60-690 mg $NaNO_3$), nitrite (0.1-0.7 mg $NaNO_2$) and nitrosamines (200-2240 ng) (Kowalski et al, (1980). Samples were measured in duplicate after the meal consumed by each volunteer. Although UDS was significantly increased in 6 out of 10 volunteers, there was no obvious correlation with dietary intake of nitrosamines, nitrate or nitrite, or with blood nitrosamine level even in one individual who consumed a meal containing 696 mg nitrate and 1.57 mg nitrite yielding salivary concentrations of 429 and 87 mg/kg respectively. However, as the authors point out, metabolic activation of nitrosamines to genotoxic derivatives occurs mainly in the liver and so short lived reactive metabolites may not have much opportunity to damage leucocyte DNA.

8.2 Epidemiology

8.2.1 Background

Numerous reviews have been undertaken on the relationship between nitrate intake and gastric cancer. In general the studies were geographical comparisons of high and low gastric cancer incidence areas or case-control studies in which the exposure to nitrate of gastric cancer control populations were compared. In both cases the variable under consideration was an estimate of exposure to enviromental nitrate or some surrogate e.g. use of well water. Geographical studies were reported from Columbia, Chile, England, Hungary, France, Italy, Denmark, China and the U.S.A. Case-control studies took place in Columbia, Chile, Japan and amongst the Hawaiian Japanese. Geographic correlational studies of nitrate in relation to oesophageal cancer have also taken place in China and Iran and in relation to liver cancer in China. There has also been consideration about the role of nitrates as a co-factor in bladder cancer.

WHO (1985) summarised the available evidence and concluded :

> "No convincing evidence of a relationship between gastric cancer and consumption of drinking-water containing nitrate levels up to 10 mg NO_3-N l^{-1}(equivalent 45 mg NO_3^- l^{-1}) has emerged . Furthermore, no firm epidemiological evidence has been found linking gastric cancer and drinking-water containing higher levels of nitrate, but a link cannot be ruled out due to the inadequacy of the data available. Gastric cancer is declining in most countries, and the risk from nitrate, if any, would appear restricted to individuals with conditions associated with low gastric acidity, rather than to the population in general.
>
> Very few studies have considered human cancers other than gastric cancer in relation to nitrates, and none of them provides convincing evidence that nitrate ingestion influences cancer incidence at other sites."

The National Academy of Sciences (1981) reached a similar conclusion:

> "Evidence implicating nitrate, nitrite, and N-nitrosocompounds in the development of cancer in humans is circumstantial. Several epidemiological studies of certain geographical/nationality groups have provided data that are consistent with the hypothesis that exposure of humans to high levels of nitrate and nitrite may be associated with an increased incidence of cancers of the stomach and oesophagus. However, in none of these studies was there a direct attempt to investigate actual exposures of nitrate, nitrite, or N-nitrosocompounds in individuals who developed cancer. In most of the studies, several other plausible causative agents were also identified."

Publications after these two critical reviews are discussed here.

8.2.2 Epidemiological Evidence for Gastric Cancer

8.2.2.1 UK

Forman et al (1985a, b) examined the incidence of gastric cancer in four regions of the UK, two of which were characterised by a high and two by a low gastric cancer incidence. The high risk regions had approximately double the mortality from gastric cancer as the low risk ones. The population examined were 200 healthy individuals attending hospitals as visitors (not for clinical attention). Each provided sputum samples that were analysed for salivary nitrate and nitrite and completed a dietary questionnaire. The low risk population had 50% higher levels of nitrate and nitrite in the saliva than the high risk populations. This difference which reflected the dietary exposure to nitrate as assessed by questionnaire, was not confounded by age, sex, social class, smoking, or the time of the last meal.

Mirvish (1985), Tannenbaum and Correa, (1985) and Forman et al (1985(b)) suggested that the results might be due to the majority of nitrate exposure in these populations coming from vegetables which would also contain anti-carcinogenic agents, particularly vitamin C. Further methodological difficulties include the adequacy of salivary assays as indicators of environmental exposure and the fact that individuals with gastritis would be able to convert nitrate to nitrite in the stomach at a substantial rate. Hence, if the high risk populations have a higher prevalence of gastritis, then their utilisation of nitrate may be far more efficient that the low risk populations. Forman et al concluded that their negative result did not mean nitrate exposure had no role in gastric cancer but it was not a rate limiting factor and could not explain the geographic distribution of the disease in the U.K.

Beresford (1985) examined the drinking-water levels of nitrate within 229 urban areas in the U.K. between 1969-73 and compared them with gastric cancer mortality rates from the same areas in the same time period. The areas covered over 90% of all British communities with populations over 50,000. She found a significant

negative correlation between gastric cancer mortality and the nitrate content of the water for both men and women, i.e. in agreement with Forman et al (1985-b). Adjusting for social factors reduced the degree of statistical significance but a negative relationship persisted. Restricting the analysis to 58 areas with moderately high water nitrate levels still resulted in a negative relationship through no longer significant.

Two problems that affect both the Foreman et al (1985-a) and the Beresford (1985) study are firstly the use of nitrate exposure data for the same time period as gastric cancer mortality data and secondly the fact that neither study covered populations where the drinking-water nitrate level exceeded the current EEC maximum acceptable level of 50 mg l^{-1}. The former problem ignores the long latent period likely to be needed between an exposure and the onset of cancer while the latter excludes communities with particularly high exposure who may have a specific risk. A study, similar in design to Beresford's but which overcame the latency problem was reported by Clough (1983). He compared water nitrate in 1946 in 43 of the local authority areas within the county of Kent with equivalent gastric cancer mortality figures for the 1959-73 period. The results showed a significant positive relationship between stomach cancer and nitrate intake for males but not for females. However the reliability of the historical water data is unknown, the small size of the areas considered means that the mortality rates are subject to random fluctuation, and it was not possible to adjust for any relevant social factors.

One way of examining the effect of high nitrate exposure in the absence of other confounding dietary factors is to examine groups of workers industrially exposed to nitrate. One such group is fertiliser manufacturers who would be exposed to nitrate containing dust. Al-Dabbagh et al (1986), followed up a group of over 1,300 male employees working for a major fertiliser producer in the North East of England. They traced all such workers employed since 1946 and compared the 304 deaths in the group with the number expected from mortality statistics for the local region. Analysis was also carried out separately for a subgroup who had particularly heavy

exposure. In neither the group as a whole nor in the high exposure cohort was there an excess of deaths from any of the forms of cancer suggested as being associated with nitrate exposure. For cancer of the stomach there were 7 and 12 deaths in the respective groups compared to 7.2 and 12.1 expected. That these men had exposure to high concentrations of nitrate was confirmed by measuring salivary nitrate in a sample of currently employed men. They had substantially raised concentrations compared to factory controls unexposed to nitrate dust, or compared to the general male population. These results confirmed those of an early study of Fraser et al (1982) in which sample census records from 1961 and 1971 were used to identify groups of fertiliser manufacturing employees throughout England and Wales. This study also showed no evidence of elevated gastric cancer rates.

8.2.2.2 Italy.

Gilli et al (1984), correlated gastric cancer incidence rates for the period 1976-79 in the 1,199 communes of the Piemonte Region of Italy with the nitrate content of the drinking water for a similar time period. They found a positive association between communes having a significantly elevated incidence rate of gastric cancer and the nitrate content of the water supply. 155 communes had water with nitrate above 20 mg l^{-1} of which 10 had elevated cancer rates. In contrast 1,044 communes had water below 20 mg l^{-1} of which only 5 had elevated cancer rates. As with the Beresford study this association is between rates from the same time period, and thus ignores a latent effect. As some of the communes are small rural areas, changing agricultural practice could have altered the nitrate content of water drastically over a 20 year period. In addition the nitrate analyses were carrried out in six different laboratories with no standardisation procedure. There is also enormous variability in the size of the communes ranging from 1.2 million inhabitants to less than 500. Estimates of cancer incidence in the small communes will therefore be extremely unreliable. The authors were unable to adjust for any social factors.

8.2.2.3 North America.

Risch et al (1985) have reported a case-control study of gastric cancer in which a large number of dietary items were considered. 246 patients from 3 areas of Canada with confirmed gastric cancer were individually matched to population controls. Both groups were asked to complete extensive food frequency and portion size questionnaire relating to the period before the onset of disease for cases and to one year previously for controls. The food items were then converted to nutrient intake data using information from a food data bank. Nitrate and nitrite were considered as separate nutrients and the daily intake of each was calculated for each individual. This is the only case-control study in which dietary exposure to nitrate and nitrite has been estimated in such a detailed way. The results show that exogenous nitrite intake, largely from preserved meat, was significantly associated with the risk of developing gastric cancer.

On the other hand nitrate intake, almost entirely from vegetables, was significantly negatively associated with cancer risk. A separate analysis shows that this apparent protective effect of nitrate disappears if it is considered jointly with ascorbate. There is no inclusion of water-borne nitrate in the analysis nor of the extent to which nitrate intake adds to the nitrite load by endogenous reduction. Also the proportion of identified cases interviewed was 44% while that for controls was 58%. The extent to which this introduces a selection bias is unknown. A further complication is that the relevant dietary factors would be likely to operate 20 to 30 years before disease onset whereas the questionnaire is concerned with the time immediately prior to it.

A case-control study of chronic atrophic gastritis, an established precursor of gastric cancer, in which measurements were taken of gastric juice nitrate and nitrite was carried out by Fontham et al (1986). This took place amongst the high-risk black population in southern Louisiana using 93 patients with proven gastritis with either metaplasia or dysplasia. The control series consisted of 78 patients who underwent gastric biopsy and were diagnosed as having

either normal mucosa or superficial gastritis. After adjusting for age and sex the control patients had higher gastric juice nitrate levels than the cases and there is a trend of decreasing nitrate along the spectrum of normal mucosa, superficial gastritis, metaplasia and dysplasia i.e. less nitrate with increased severity of the lesion. Gastric juice nitrite however showed no relationship with disease although, in both cases and controls, the levels were substantially higher if the gastric pH was greater than 5.0. The authors also report a comparable study from a group of patients from Narino, the exceptionally high incidence area in Colombia, South America. Gastric juice nitrate levels were significantly higher than those in Louisiana blacks and showed a positive relationship with severity of disease. A similar trend was seen for gastric nitrite levels when the pH was above 5.0. The disparity between the two sets of results was suggested to be due to different sources of nitrate, mainly water, grains, and root vegetables in Colombia and green leafy vegetables in Louisiana. The protective factors in the latter and may therefore outweigh any effect of nitrate. A substantial nitrate exposure with low vitamin intake could explain the high incidence rates in Colombia but there is no evidence that nitrate parameters play any role within the Louisiana population.

8.2.2.4 Japan.

A comparison of 24-hour urine samples from 52 residents of Akita and 52 residents of Iwate , respectively high and low incidence areas for gastric cancer in Japan, was carried out by Kamiyama et al 1987. The nitrate levels in the urine were approximately 40% higher in the low risk area where the gastric cancer rates were about half those in the high risk area. The urinary nitrate level was highly positively correlated with the consumed amounts of vegetables, in particular pickled vegetables. The same subjects also took part in a test to examine their propensity to synthesise N-nitrosoproline endogenously following a loading dose of proline. This test has been developed as a generalised assay for individual ability to synthesise N-nitrosocompounds (Ohshima and Bartsch, 1981). Interestingly the high risk population could synthesise over

75% more N-nitrosoproline than the low risk population given a standard loading dose and despite the reduced total nitrate intake of the high risk population. Further analysis of this study is currently taking place.

8.2.2.5 China.

A similar study was carried out by Lu et al (1986) in China. In this case the comparison was made between areas which differed in their risk of oesophageal cancer. 24-hour urine samples were collected after a loading dose of proline from a total of 238 healthy subjects living in Lin-xian (high risk) or Fan-xian (low risk). There was a five-fold difference between these two areas in the mortality rate of oesophageal cancer. The high risk subjects showed a statistically significant elevation in their urinary nitrate and N-nitrosoproline. The diet of Lin-xian residents was reported to be high in pickled vegetables, low in fresh green vegetables, and their drinking water contained high levels of nitrate.

8.2.2.6 Other gastric cancer studies

Case-control studies of gastric cancer have also been reported from Greece (Trichopoulos et al, 1985), Poland (Jedrychowski et al, 1986) and the U.S.A. (Tajima et al, 1985; Correa et al, 1985). None of these estimated exposure to nitrate in the same way as the Canadian study but analysed differences between cases and controls in terms of other food constituents. There was considerable diversity in the risk identified in these studies, most of which are not relevant to this review. However it is noteworthy that all the studies, except the Japanese one, find a significant protective effet of vitamin C or food containing it in high quantities.

8.3 Summary

Nitrate is not mutagenic.

Recent epidemiological research provides no evidence that nitrate induces cancer in man.

9. EVALUATION

The subject "Nitrate in drinking water" may at first glance appear an easy topic, simple and well reviewed. It has proved to be very complex, and there are parts where present knowledge is inadequate and incomplete. Previous scientific and administrative conclusions and decisions are in need of revision, and further scientific and development work is required. In particular we point to the following matters :

1. The rising nitrate concentration in some European waters may cause concern. While the extent of this rise is well known for some countries, there are still important areas (mainly in Southern Europe) where little or no information has been published. This paucity of information across Europe makes it difficult to evaluate problems and subsequently the consequences of strict implementation of present water quality criteria.

2. Whilst pilot plant process data for nitrate removal from drinking water is available there is lack of experience with full scale operation. Some nitrate removal processes bring about further changes in the composition of the treated waters that might cause new problems in both health and distribution; malfunction of nitrate reduction treatment plants would cause water contamination. We therefore advise a cautious advance in such water purification programmes.

3. Nitrate migration to surface and ground water is closely connected with land use and agricultural methods. Forecasts on potential water nitrate levels are based on historical agricultural practices. The latter are changing and the consequences difficult to predict. We note that no impact analysis connecting nitrate water standards and agricultural practice in Europe is available.

4. Nitrate leakage should be reduced to a practicable minimum by making use of the principles of good agricultural practice. However, these principles must be adjusted to suit local conditions such as soil, climate and agricultural realities. Agricultural development work should focus upon minimising nitrate leaching although only in the long run can a solution be expected.

5. While it is now well established that nitrate synthesis is a normal process in the human body, more information is needed on its extent, and especially on the circumstances under which this synthesis is enhanced.

6. The current information available on the nitrite formation and fate in the human body is incomplete. Nitrate must be converted to nitrite before it exerts a biological effect and the lack of detailed knowledge about nitrite and nitrate balances in man adds to the difficulties of interpreting data.

7. There is a general agreement that infant well-water methaemoglobinaemia is a multifactorial disease, but little detailed information is available on the factors involved and their relative importance. The evidence available indicates that the nitrate content of the water is of minor importance, at least when the concentration is below 100 mg NO_3^- l^{-1}. As the condition has virtually disappeared in Western Europe it is very difficult to research the subject. Further detailed investigations on the Hungarian epidemics might be helpful.

8. The direct causal relationship between the occurrence of well-water methaemoglobinaemia in infants and the nitrate content of water has been overstated. The numerous instances in which infants have received high nitrate containing water without adverse effects and the poor hygienic quality of the water involved in the cases described indicate that bacterial contamination of the water (which is generally reflected by a high nitrate level) and the occurrence of gastroenteritis are the most likely causes. This viewpoint is strengthened by the fact that infantile methaemoglobinaemia has not been reported when neonates have been exposed to mains piped water, the major source of drinking water for the Western European population, except when the water has been contaminated by bacteria.

9. The published epidemiological research clearly indicates that nitrate intake is not closely associated with human cancer incidence.

10. In the opinion of ECETOC the reductions by the WHO in the upper

allowable concentration for nitrate in drinking water from 100 mg NO_3^- l^{-1} to 45 mg NO_3^- l^{-1} (10 mg NO_3-N l^{-1}) were not justified for two reasons. Firstly the clinical information shows infantile methaemoglobinaemia level associated with nitrate concentrations of 50 - 100 mg NO_3^- l^{-1} to be within the normal range. Secondly they did not attribute sufficient importance to gastro-enteritis and the poor hygienic quality of the drinking water involved in the few infantile methaemoglobinaemia cases attributed to low nitrate concentrations (below 100 mg NO_3^- l^{-1}). The evidence available coupled with the absence of clinical cases in Western Europe supports the earlier recommendations.

11. We can find no evidence to support the need for a guide level of 25 mg NO_3^- l^{-1}, the current requirement of the EEC.

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TABLE 1

Range of Mean Values for Nitrate and Nitrite Contents of Dairy and Meat Products

	Total No. of Samples	Range of Values NO_3^-	Range of Values NO_2^-	References
Dairy Products				
Liquid Milk	-	</.0-8	<0.2	1,2,3,4
Yoghurt	-	<3.0	<0.33	4
Condensed Milk	-	<3.0	<0.2	4
Cheese	-	<3-27	<0.2-1.7	1,4,5
Meat Products				
Fresh Meat	56	<2.7-53	<0.2-13	4,7
Preserved Meat	24	14-36	3.4-26	4
Canned Corned Beef	38	44	10	7
Pork Luncheon Meat	34	78	6	7
Raw Ham	171	149-343	14-21	4,5,6,7
Cooked Ham	49	945	14.5	4
Bacon	14	56-172	27-59	7
Sausage/Sausage Meat	386	14-489	</-64	4,5,6,7

References

1) Denmark SFI (1981)
2) Burg P.F. van (1986)
3) White J.W. (1975)
4) Collet P. von (1983)
5) Deschamps P.(1980)
6) Custot et al,(1976)
7) Fudge and Truman (1973)

TABLE 2

Nitrate Content of Various Vegetables (Corré and Breimer, 1979)

Nitrate Concentration Mostly Lower than :			
200 mg/kg	500 mg/kg	1000 mg /kg	2500 mg/kg
asparagus chicory garden bean green pea mushroom potato sweet pepper sweet potato tomato	broccoli cauliflower cucumber eggplant gherkin melon onion scorzonera turnip	cabbage (white, red and savoy) carrot curly kale french bean parsley (root)	cabbage (oxheart) celeriac endive garden cress leek parsley (leaves) rhubarb turnip cabbage
Nitrate Concentration Frequently Greater than 2500 mg/kg			
beetroot celery chervil lambs's lettuce lettuce purslane radish and black radish spinach turnip tops			

TABLE 3

Dietary Intake of Nitrate and Nitrite by Duplicate Portion Technique

(mg/person/day)

Nitrate		Nitrite		Country	Reference
Mean	Range	Mean	Range		
131.2	18.2 - 574	5.1+	1.3 - 40.2	Holland (c)	Stephany & Schuller (1978-1980)
109.9	ND	2.8	ND	Holland (d)	
47.8	25.6 - 80.7	3.0	0.5 - 5.9	Sweden	Jagerstad & Nilsson (1976)
43 (a)	9.0 - 76.0	2.4++	1.0 - 4.6	) F.R.G.	Kampe (1984)++
71 (b)	22.1 - 129.7	2.3++	1.3 - 3.9	)	
116.4	-	1.2		Italy	Cocchioni et al, (1986)

a) Normal diet
b) Normal diet incorporating home garden produce
c) 100 "summer" diets) for white colar workers
d) 201 "summer + winter" diets)

ND = No Data
\+ = possibility that values may be elevated due to analytical difficulties
++ = data supplemented by personal information.

TABLE 4

Analysed (A) and Calculated (C) Estimates of Daily Intake for Nitrate and Nitrite (mg/person/day)

NO_3	NO_2	Basis of Estimate	Country	Author	
110 (a)	>2	C	France	Causeret (1984)	
49.3	1.72	C	Germany	) Selenka and	
55.95	2.5-3.9	A	Germany	) Brand Grimm) (1984)	
31.4	1.17	C	Norway	Cislason and Dahle (1980)	
			Poland		
130(b)	No Data	A	Adults		
104(c)	2.65 (c)	A	Children 1-3 Y	) Nabrzyski and	
21.8	0.94	A	Infants .25-1 Y	) Gajewska (1984)	
91	No Data	C	Switzerland	Trempa (1980)	
74.4	No Data	C	UK	Royal Society UK (1983)	
64-297	No Data	A	UK	Chilvers et al (1984)	
71	No Data	C	UK	Lindsay and Crossett (1984)	
100	2.6		US	White (1975, 1976)	
39	.34	C	US	FDA (1979) (d)	
70	.54	C	US	Hartman (1982) (d)	
100	.57	C	US	Birdsall (1981) (d)	
75	.77	C	US	NAS (1981)	Average diet
78	1.7	C	US	NAS (1981)	High cured (e) meat diet
268	0.77	C	US	NAS (1981)	Vegetarian diet (f)
233	0.77	C	US	NAS (1981)	Nitrate rich (g) water diet

a Based on nitrate free water; 35 mg l^{-1} NO_3 in water doubles total NO_3 Intake.
b From meals prepared in a students canteen.
c From meals prepared in a childrens home.
d Quoted by NAS (1981).
e Assumes 4 x average daily consumption of cured meats.
f Assumes 4 x consumption of average vegetables in same proportions.
g Uses data for the Sangamo River area, Illinois (greater 100 mg NO_3/1).

TABLE 5

The Contribution of Various Foods to the Nitrate Content of the Total Diet (%)

Food Types							Country	Author
Cured Meat	Vegs	Milk Prod	Cereal Prod	Fruit Juice	Bevs	Water		
9	91	ND	ND	ND	ND	ND	France	Causeret (1984)
18.5	72.5	0.7	5.5	2.9	ND	0	Germany	Selenka et al, (1976)
7 9	84 83	ND ND	3 4	6 4	ND ND	+	Germany (1) (2)	Kampe et al, (1983)
ND	ND	ND	ND	ND	ND	ND	Poland	Nabrzyski et al, (1984)
ND	100	ND	ND	ND	ND	ND	Norway	Gislason et al, (1980)
6.25	70.0	0.25	1.6	1.05	ND	20.9	Switzerland	Trempe (1980)
7	54.5	17.6	ND	ND	ND	20	UK	Royal Society UK (1983)
2.2	67	1.5	2.8	3.0	9.1	14.1	UK	Lindsay & Crossett (1984)
9.4	86.3	0.2	2.0	1.4	ND	0.7	US	White (1975, 1976)
0	95	ND	ND	ND	ND	5	US	F.D.A (1979)[3]
0.6	91.7	0.3	2.8	1.8	ND	2.8	US	Hartman (1982)[3]
9.4	86	0.2	2.0	1.4	-	0.7	US	Birdsall (1981)[3]
1.6	87	0.2	1.6	6	ND	2.6	US	NAS (1981) Average diet
6.0	83	0.2	1.5	6	ND	2.5	US	NAS (1981) High cured meat diet
0	97	0.1	0.5	1.8	ND	0.7	US	NAS (1981) Vegetarian diet
0.5	28	0.1	0.5	1.8	ND	68	US	NAS (1981) Nitrate rich water diet

1. The papers shows a significant intake of 28 mg/NO_3/person/day in drinks measured for 20 families.
2. These appear to be six families out of the 20 mentioned in 1. The principle nitrate sources being included in water or fruit juices.
3. Quoted from NAS (1981).

\+ The drinking water contained 50 mg NO_3^- l^{-1}.
ND = No Data.

TABLE 6

Mean Nitrate and Nitrate levels in human gastric juice

	NO_2^-	NO_3^-	Reference
	(mg/l)		
Acidic Juice pH <4 d			
Fasting	0.05		Kyrtopoulos, 1985
	0.6		Walters et al, 1976
	0.08		Ruddell et al, 1976
	0.1	6.6	Mueller et al, 1986
After meal	13.8		Walters et al, 1979
Over 24 h period	0.05-0.9		Milton-Thompson et al, 1982
Hypochlorhydric "normals" (fasting)			
pH 7.0	1.2		Ruddell et al, 1976
pH 5.6-6.4	0.7	7.5	Mueller et al, 1986
pH 6.5-7.6	1.4	11.7	Mueller et al, 1986
Patients with GI disease			
Pernicious anaemia	2.7	13.0	Stockbrugger et al, 1984
" "	2.2	2.2	Dolby et al, 1984
Hypogammaglobulinaemia	6.2	8.9	Dolby et al, 1984

TABLE 7

Normal Infantile Methaemoglobin Levels
(Including values given as background levels in section 7.2.3)

Methaemoglobin %	Age	Remarks	References
2.2 ± 1.1 1.5 ± 0.81 1.2 ± 0.78 0.82 ± 0.63	0 - 72 d 0 - 10 d 1 - 12 m adults	42 prematures 25 matures 8 matures	Kravitz et al, 1956
1.0 - 1.3 0.8	0 - 90 d older infants	)89 infants)	Simon et al, 1964
1.6 (range 0-2.9)	Not given	63 infants	Winton et al, 1970
1.30 (mean) 1.24 (mean)	1 - 60 d 61 - 90 d	96 infants 75 infants	Shuval and Gruener (1972)
2.7 (median) 2.2 (median) 1.9 (median)	30 - 60 d 61 - 90 d 91 - 120 d	194 samples 100 samples 70 samples	Shearer et al, 1972 Goldsmith et al, 1975
0.89 (mean)	7 d - 10 m	87 infants	Gruener and Toeplitz, 1975
0.29 ± 0.05	Not given	32 infants	Pilawska, 1976
0.56 (mean)	mean: 15 m	92 children	Fernicola and Azevado, 1980
0.68 ± 0.22 0.60 ± 0.20	0 - 7 d 8 d - 1.5 y	30 infants 30 infants	Hegesh and Shiloah, 1982

We suggest that the differences between authors reflect methodological rather than real differences. The 'normal level' as found by various authors appears to be ill defined, and partly to be dependent on the analytical procedure. The WHO (1985a) suggests that a methaemoglobin level of 1-2% can be considered normal. As values below this are also quoted we suggest a normal range of 0.5-2% but it appears that the normal range could be somewhat broader than this.

TABLE 8

Well water methaemoglobinaemia with water NO_3^- concentrations less than 100 mg/l.

					Illness			Water			Remarks
Reference	Country	Year	N°. of cases	Age days	Preceeding illness	Hospital remarks	Meth. level %	Supply	Hygienic conditions	NO_3^- mg/l	
Scott (1948)	USA	'47	1	22	Respiratory	Cyanosis	10	Well	NI	14?	Cyanosis developed shortly after infant was given acetophenetidin and acetylsalicylic acid. Probably 14 mg NO_3^--N l^{-1}.
Donahoe (1949)	USA	'48	1	49	NI	Cyanosis, recurrent attacks stopped when water source changed	NI	Well	Coli present	50	
Robertson and Riddell (1949)	Canada	'49	1	NI	NI	Cyanosis	NI	Well	NI	75	
Walliker and Baxter (1949)	USA	'46	2	13	None	Rapidly developing cyanosis	55	Well	NI	60	Probably NO_3^--N rather than NO_3^-, but unclear.
				60	Pale irritable, then sudden cyanosis		59	Well	NI	90?	

					Illness			Water				
Reference	Country	Year	N°. of cases	Age days	Preceeding illness	Hospital remarks	Meth. level %	Supply	Hygienic conditions	NO_3^- mg/l	Remarks
Frith (1950)	USA	'50	1	60	NI	Recurrent cyanosis	NI	Well (shallow)	Near manure and fertilizer storage, unhygienic	33?	Similar cases mentioned. No additional details Water sampled 19 days after.
Ewing and Mayton-White (1951)	UK	'50	1	32	Vomit day before	Cyanosis	45	Well	Heavily contaminated by bacteria. E Coli >180/100ml	95	Water was boiled before use, may have increased concentration.
Richard (1951)	France	'48	1	41	None	Cyanosis had gradually develped over 24 days	+	Well (water boiled)	NI	58	0.5 mg NO_2^-/1. The infant drank 7 portions, each 90 g, per day.
Armengau (1954)	France	'53	1	NI	NI	Cyanosis	>11	Well	Contaminated by bacteria, E Coli >25/1	"More than 70"	
Bodo (1954)	Hungary	'54	1	14	Diarrhoea	No details	+	Well	NI	56	Stomach sterile, but E Coli in duodenum. Previously given ultraseptyl by injection. Mixed feed
Betke and Kleinhauer (1957)	FRG	56	1	10	Dyspepsi + ear and throat infection	Cyanosis developed when dyspepsi was on retour	47	Community well	NI	33-552	Nitrate content variable. Both measurements given.

					Illness			Water			
Reference	Country	Year	N°. of cases	Age days	Preceeding illness	Hospital remarks	Meth. level %	Supply	Hygienic conditions	NO_3^- mg/l	Remarks
Wedemeyer (1956)	FRG	56	3	19	None	Acute rapidly developing cyanosis resistant to treatment. Death.	+	Well	Strongly contaminated with bacteria	approx 40	May have had hereditary methaemoglobinaemia
				81	Vomit	Acute rapidly developing cyanosis	NI	Well	Strongly contaminated with bacteria	approx 40	Further water analysis later indicated that these wells had nitrate concentrations that varied greatly
				40	None	Cyanotic condition developing over a period of 3 days	30	Well	Strongly contaminated with bacteria	approx 25	
Horn (1958)	DDR	'58	1	60	Diarrhoea for 8 days	Cyanosis	+	Well	24 mg/l of organics	89	
Verger et al (1960)	France	'58	1	21	Diarrhoea (green)	Cyanosis	+	Well	NI 0,35 mg NO_2^-/l	50	The infant was on acidified milk and mineral water. Cyanosis came after changing to a diarrhea diet and rice water, made with well water.

					Illness			Water			
Reference	Country	Year	N°. of cases	Age days	Preceeding illness	Hospital remarks	Meth. level %	Supply	Hygienic conditions	NO_3^- mg/l	Remarks
Thal et al (1961)	GDR	'59?	4	10 38 56 66	Dyspepsi (One case with E. Coli 026)	Quickly developing cyanosis (over a few hrs)	68 46 72 68	Well	NI	80 78 50+ 95	+ Determined some time after the incident
Vigil et al. (1965)	USA	'62	1	28	Diarrhoea	Cyanosis	+	Municipal Water	Polluted	63	The water was boiled and concentrated, approx. 73 mg NO_3 l^{-1}. The disease relapsed in hospital after the treatment, which had to be repeated.
Bailey (1966)	USA	52-66	40	7-280	Diarrhoea (all cases) Anorexia, temperature, vomiting.	Cyanosis All cases had a peculiar odour of decaying vegetation	NI	Pond, cistern and wells	Hygienically bad, with remains of decaying animals etc.	55-110	A review of 40 cases treated at a hospital 52-66 individual case descriptions not given. It is not clear if the water analysis is on N or on NO_3^- basis.
Miller (1971)	USA	'69	1	30	None	Cyanosis	50	Well	40 ft from overflowing cesspool, but no bacterial contamination found.	77	

					Illness			Water			
Reference	Country	Year	N°. of cases	Age days	Preceeding illness	Hospital remarks	Meth. level %	Supply	Hygienic conditions	NO_3^- mg/l	Remarks
Swiatkowska and Gockowska	Poland	'80	3	35	NI	Coma, Cyanosis	46	Well	NI	46	Readmitted later (conditions unspecified) with 2,8% methaemoglobin level. This went down when water source changed.
				35	Diarrhoea, Vomiting	Cyanosis, limp	+	Well	NI	86	Readmitted 2 weeks later with diarrhea and 13% methaemoglobin level after discharge 1 month later the level was 0,9%.
				60	NI	Very serious conditions (not clearly specified) Pulse 180 per min, dull heart sounds, enlarged liver, general cyanosis	10	Well	The water was murky and foul tasting	35	
Farre Sostres et al. (1982)	Spain	'80		9		see table 13		Well	NI	73	Epidemic. Details see Table 13.

NI = No information

\+ = No quantitative methaemoglobin measurements, but brown blood colour or similar qualitative observation.

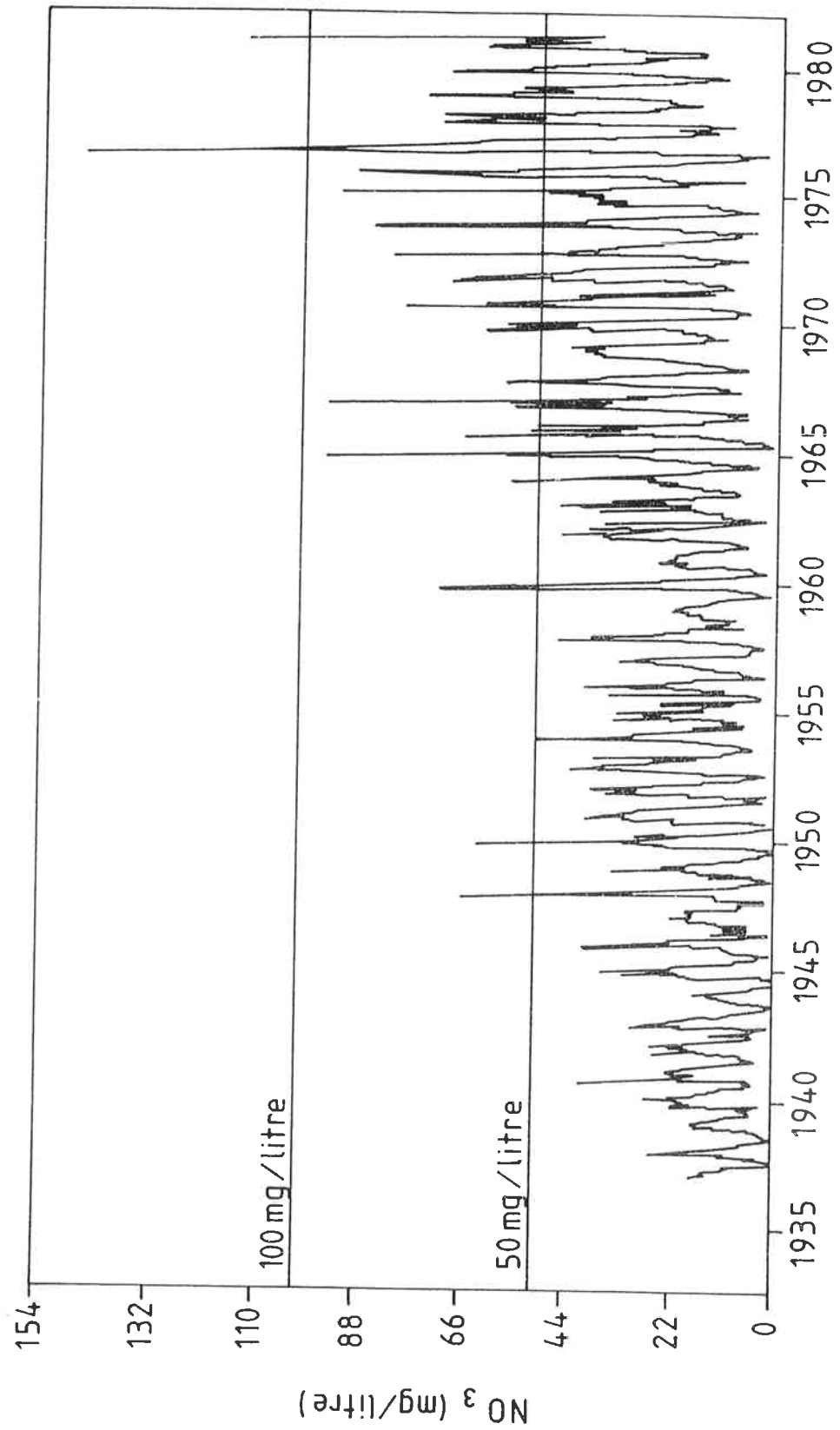
TABLE 9

Clinical and laboratory findings in a enteritis epidemic in a spanish community where 9 of 50 infants developed methaemoglobinaemia, and 7 of these had visible cyanosis. Methaemoglobin concentrations was estimated from the colour of blood (Farre Sostres et al. 1982).

Age	Clinical Findings	Analytical Findings
With cyanosis :		
23d	Cyanosis (light) Diarrhoea Rhinitis Conjunctivitis Thrush*	Leucocytosis Metabolic acidosis Hypernatriemia
31d	Cyanosis Diarrhoea Thrush	Metabolic acidosis Anaemia
26 d	Cyanosis (light) Diarrhoea Rhinitis	Metabolic acidosis Hypernatriemia Hyperkalemia
24d	Cyanosis (light) Diarrhoea Rhinitis Conjunctivitis Thrush	Metabolic acidosis
15d	Cyanosis Diarrhoea Thrush	Low diaphorase
48d	Cyanosis (mild) Diarrhoea	
32d	Cyanosis (mild) Diarrhoea Mild dehydration Thrush	Metabolic acidosis Anaemia
Without cyanosis :		
4m/2d	Conjunctivitis	-
5d	Diarrhoea Thrush	-

* Thrush = infections associated with Candida albicans

FIGURE .1.

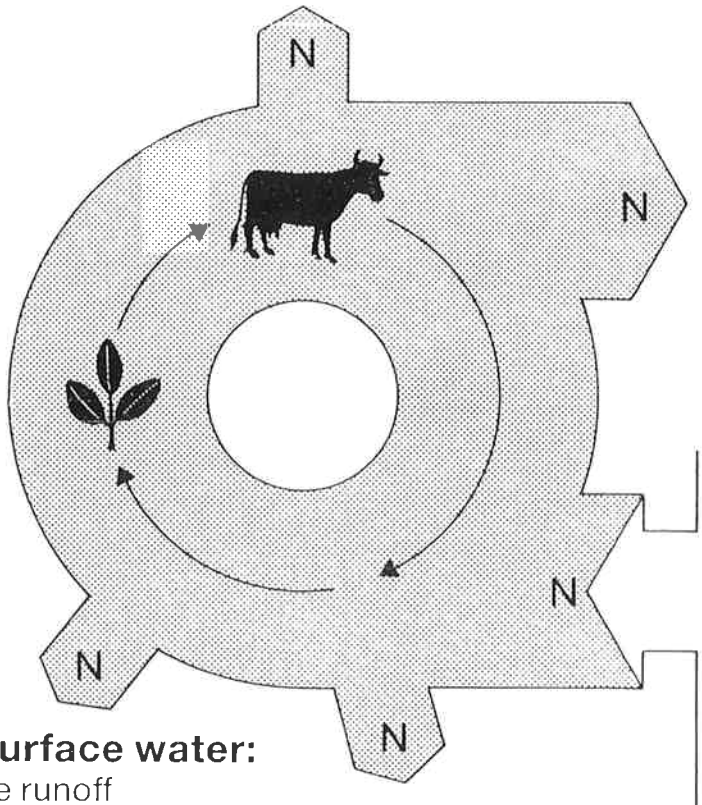


NITRATE IN THE RIVER STOUR AT LANGHAM

Nitrogen cycle on a farm

(after OWEN and Sigrid JÜRGENS-GSCHWIND, 1986)

Into the atmosphere:
Denitrification, volatilization
loss to atmosphere



Into yields:
Field crops,
animal products

Atmosphere:
Rainfall,
biological fixation

Soil:
Breakdown of
organic matter,
mineralization,
soil solution

Agriculture:
Farmyard manure,
mineral fertilizers

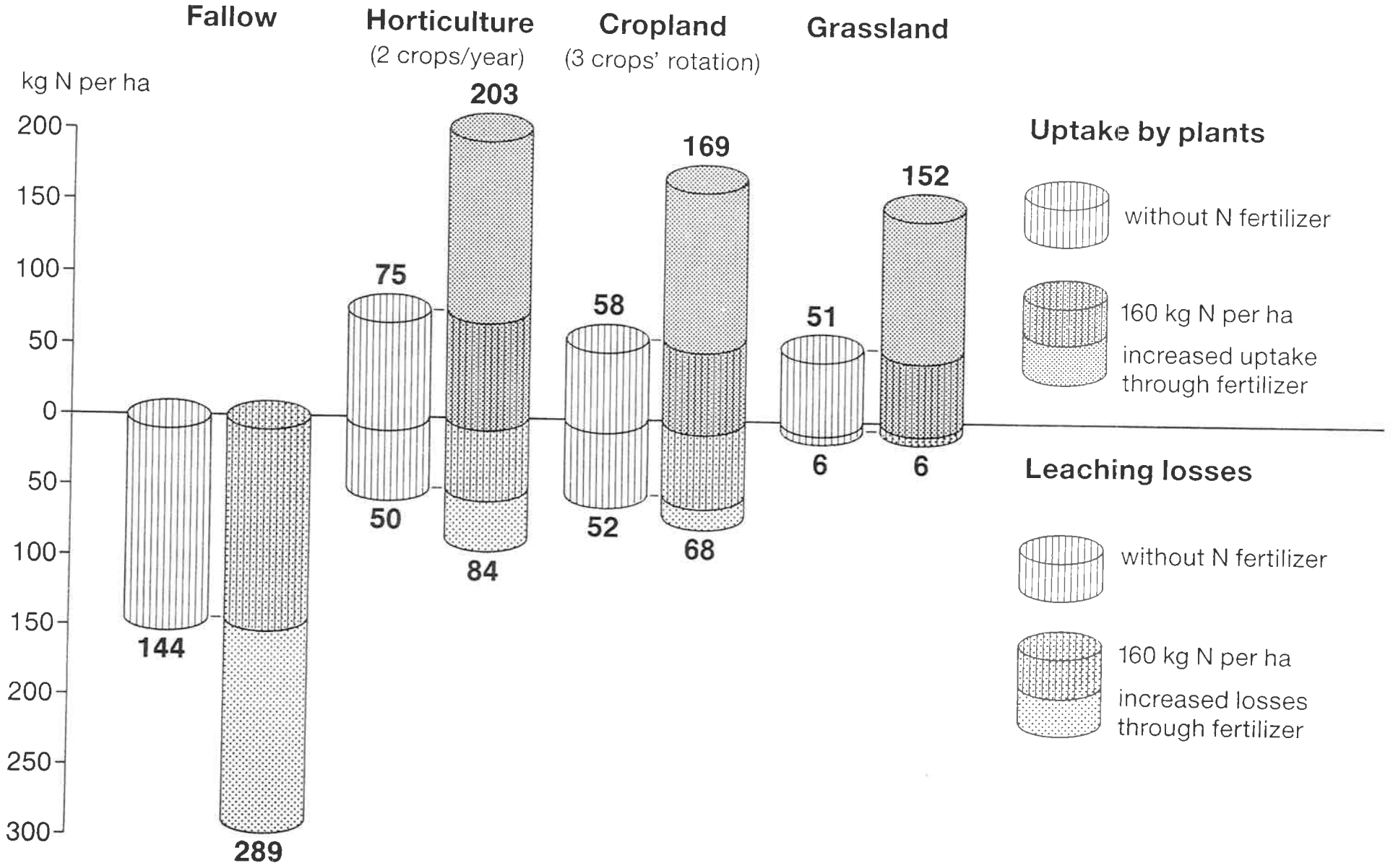
Towns:
Household waste,
urban composts

Into surface water:
Surface runoff
and soil erosion
into surface water

Into the soil:
Leaching
into surface
and groundwater

The effect of crop rotation on the uptake of nitrogen and its loss due to leaching

(Mean of 11 years, after DRESSEL and JUNG, 1984,modified)



APPENDIX 1

Glossary

Achlorohydric : (hypochlorohydric)	deficiency of hydrochloric acid from maximally stimulated gastric secretions.
Aetiology :	causes or origins of a disease.
Anaemia :	a reduction below normal in the composition of red blood corpuscles.
Atrophic gastritis :	condition in which the thickness of the stomach mucosa is greatly reduced with complete or almost complete disappearance of gastric glands and replacement by mucous secreting epithelium.
Chronic pneumonitis :	long lasting inflammation of the lung.
Duodenum :	first portion of the small intestine, into which the stomach empties.
Dysplasia :	Condition of abnormal development: alteration in the size, shape and organisation of adult cells.
Dyspnoea :	difficulty in breathing, shortness of breath.
Ileostomy :	surgical technique to exteriorise the lower part of the small intestine.
Enteritis :	inflammation of the intestines, especially the mucosal surface.
Extra medullary :	outside the inner portion of an organ, e.g. bone, marrow, brain, spinal cord.
Foetotoxic :	toxic to the foetus of the animal in the uterus.

Gammaglobulinaemia :	deficiency of γ globulins in the blood.
Gastric resection :	surgical removal of part or all of the stomach.
Gavage :	feeding by stomach tube.
Globulinaemia :	deficiency of globulins in the blood.
Haematopoiesis :	the process of formation of red blood cells.
Histopathologic :	microscopic changes in tissues resulting from injury or disease.
Histolytic sarcomas :	degenerating tumour derived from connective tissue.
Hypo γglobulinaemia :	deficiency of γglobulins in the blood.
Interferon :	protein produced naturally in the body, active against many viruses.
In Vitro :	in the test-tube.
In Vivo :	in the living organism.
Lambliasis :	infection with Giardia lamblia organisms, producing protracted, intermittent, diarrhoea.
Leguminous :	pertaining to leguminosae, pod bearing vegetables e.g. peas, beans, having root nodules capable of fixing atmospheric nitrogen.
LPS :	lipopolysaccharide, chemical component of gram-negative bacterial cell walls.
Lympo-reticular :	pertaining to the lymphoreticular system, i.e. a network of vessels and cells responsible for conveying lymph, removing particulate material from the body, and taking part in the immunological reactions.

Macrophage :	large phagocytic cells found in blood and connective tissue, removes foreign material or cells.
Malignant : lymphomas	malignant tumour of lymphatic tissue.
Metaplasia :	Change in the types of cells in an adult tissue to a form not normal for that tissue.
Mucosa :	mucous membrane, lining of various tubular cavities of the body, with glands secreating mucous.
Nephrosis :	degeneration of kidney tissue.
Oedema :	pathologic accumulation of fluid in tissue spaces.
Peritoneum :	membrane lining the viscera and the abdominal wall.
Peritoneal :	the potential space between the layers of the peritoneoum lining the viscera and the abdominal wall.
Pernicious : anaemia	anaemia characterised by large red blood cells and the occurrence of achlorohydria caused by malabsorption of vitamin B_{12}.
Plasmocytosis :	increased number of plasmocytes in tissues.
Tissue anoxia :	deficient oxygen suppply or uptake in a tissue.

APPENDIX 2

NITRATE CONCENTRATION OF EUROPEAN DRINKING WATER, RIVER AND GROUNDWATER.

European drinking water is derived from both surface water and groundwater sources. The proportion of these sources varies from country to country (Table 1). The values given in Table 1 refer to piped public water supplies.

A small percentage of the European population (but a significant number of people) is supplied from small private wells. In many cases these are shallow, located in agricultural areas, and are at risk of being polluted by contaminated surface water caused by poorly developed facilities for disposal of human and animal waste (WHO, 1985).

The use of water of poor hygienic quality has been a major factor in the occurrence of disease. Hence the desire to replace such shallow water wells by piped, bacteriologically safe, public water supplies.

The following paragraphs summarize the recent data for most European countries; in many cases care is required when drawing conclusions due to the fragmentary nature of the original data.

Belgium

Surface Waters

R. Meuse nitrate concentration varies between 2-14 mg NO_3^- l^{-1} (average 6 mg NO_3^- l^{-1}) at the Belgian/French border, and between 9-16 mg NO_3^- l^{-1} (average 13 mg NO_3^- l^{-1}) at the Belgian/Dutch border.

Lowland/coastal areas with intensive agriculture and cattle and pig-breeding - small streams reach 100 mg NO_3^- l^{-1} in winter; exceptional values of 800 mg NO_3^- l^{-1} have been observed.

Groundwaters

Ardennes region, 10-15 mg NO_3^- l^{-1}.
Bruxellian Soil, up to 100 mg NO_3^- l^{-1} but blended.

Agricultural areas (South of Brussels), 20-50 mg NO_3^- l^{-1} but increasing. Private wells, 40% of wells have nitrate concentrations greater than 50 mg (regional study).

Drinking water

During 1979-1982, the average concentration of nitrate in public water supplies was about 11 mg NO_3^- l^{-1}. Only 0.7% of samples exceeded 44 mg NO_3^- l^{-1} and in about 11% of samples the concentration was between 22-44 mg NO_3^- l^{-1}. Extrapolated data indicates that about 456,000 persons from the whole Belgium population were exposed to nitrate concentrations above 50 mg NO_3^- l^{-1}.

5-10% of the population still depend on shallow private wells of which it is estimated 25% supply water at concentrations greater than 50 mg l^{-1} (Brabander and de Labeau, 1987). The main reasons are considered to be widespread use of animal manure, and in most cases the shallow construction of wells.

Bulgaria

In 1982, about 4% of the inhabitants in 243 settlements (375,594 persons) were exposed to water with nitrate levels above 50 mg NO_3^- l^{-1}, and about 0.7% (68 settelements, 65,912 persons) were exposed to levels above 100 mg NO_3^- l^{-1}. At present, about 4% of the total population of 8.8 million is exposed to nitrate levels of 50 mg NO_3^- l^{-1} or above (WHO, 1985).

Czechoslovakia

Most of the drinking water is derived from surface waters increasingly polluted by nitrates from agricultural sources. Based on present estimates, about 1.8% of the total population of about 8 million receives drinking water containing more than 50 mg NO_3^- l^{-1}.

Denmark

The output of all waterworks supplying more than 10.000 m^3/year (accounting for more than 99% of the drinking water in Denmark) has been analysed for nitrate (Anon, 1984). For the country as a whole 8% of the investigated

waterworks supplying about 6-7% of the Danish population (total population is 5.1 m) exceeded 50 mg NO_3^- l^{-1} either temporarily or permanently, and 18% of them exceeded 25 mg NO_3^- l^{-1}.

Problems are reported with shallow private wells that are at risk from direct pollution by surface water.

France

Drinking water

Table 2 shows the number of people supplied with certain mean nitrate concentrations, and the number of distribution systems involved, for the country as a whole (Anon, 1979-81).

The highest nitrate levels are found in the northern and western parts of the country, particularly in groundwater in highly developed agricultural areas. About two thirds of the population is served by groundwater, and one third by surface water.

Federal Republic of Germany

Surface Water

Figure 1 illustrates nitrate data for several major German rivers over a period of 20 years (Bundesanstalt für Gewässerkunde, Koblenz und Uwelttbundesamt, Berlin, 1986/87). From this it appears that the increasing concentration has halted (for the Danube), or even decreased (Mosel, Weser, Elbe).

Groundwater

73% of drinking water is derived from groundwater. The nitrate concentration of the ground water varies greatly from region to region depending on depth of groundwater table, land use, and population density. Table 3 shows the nitrate content in drinking water wells of the official water authorities in the Federal Republic of Germany in 1982/83 (Aurand, 1985). Recent data showed 73% of the produced drinking water to contain less than 25 mg, 24% to contain 25-50 mg, and 6% to contain (permanently or only periodically) more than 50 mg nitrate per liter (DLG - German Agricultural Society Colloqium, 1987) e.g. in Northrine-Westphalia higher

nitrate levels have been reported. In Schleswig-Holstein, with intensive cereal cropping, in 1984 95.1% of the piped drinking water contained less than 50 mg NO_3^- l^{-1} and 1% more than 90 mg NO_3^- l^{-1}. However, a study of 5,595 private wells - 40% of which lay in the sandy, permeable "Geest" soils - showed only 71.3% to have less than 50 mg NO_3^- l^{-1}, 16.3% to have 51-90 and 12.3% to have more than 90 mg NO_3^- l^{-1} water (Länderarbeitsgemeinschaft Wasser, 1986). Higher nitrate concentration in shallow well-water is quite common and mostly due to bad hygienic conditions (bacterial growth). A 1980 study of 330 rural districts showed that 9% of the population received water with a nitrate concentration in the range 25-250 mg NO_3^- l^{-1}.

Hungary

Groundwater accounts for about 90% of public water supplies, of which about half is bank-filtered surface water. About 7% of the total publicly supplied water contains nitrate around or above the Hungarian guideline level of 40 mg NO_3^- l^{-1}.

Severe problems exist in small settlements where private or individual wells are dug to the shallow aquifer. Around 316,000 people are served by 944 private wells, of which nearly 500 have very serious nitrate problems, 200 have frequent problems, and 200 have occasional problems with nitrate. A number of these private wells will be replaced by public supply.

Ireland

About 85% of the population of approximately 3 millions is served by waterworks deriving water from surface water sources. Small, private wells have only a few problems. Slight increases in the nitrate concentration of the larger rivers have been reported (Anon, 1983) but they remain well below recommended limits.

Italy

About 100,000 people are supplied with groundwaters of higher nitrate content in intensive agricultural areas of the Marches, Emilia - Romagna and Piemont. Maximum nitrate concentrations in drinking water of almost 100

mg NO_3^- l^{-1} have been reported. In many cases blending options are available to produce drinking water with nitrate concentrations of 25 - 30 mg NO_3^- l^{-1}.

Luxembourg

The main groundwater source is the well-protected sandstone aquifer in central Luxembourg; the nitrate concentration ranges between 10 and 40 mg NO_3 l^{-1}. However, the nitrate content of wells situated in agricultural areas is increasing and about 1800 people (0.5% of total population) receive supplies exceeding 50 mg NO_3^- l^{-1}.

Netherlands

Surface Water

About one third of the population of 14 million is served by waterworks using surface water. Some large waterworks using the rivers Rhine and Meuse (containing about 15 mg NO_3^- l^{-1}) produce very low nitrate concentration in the treated water due to denitrification during recharge in dune areas.

Groundwater

About two thirds of the population derive drinking water from groundwater sources. Increases in nitrate concentrations are attributed to the dumping of slurry from intensive cattle rearing, and it is estimated that 58 of the 240 aquifers will experience elevated nitrate concentrations.

About 0.1% of the population is served by private wells, of which probably over 50% have nitrate concentrations exceeding 50 mg NO_3^- l^{-1}, and an appreciable number have concentrations well over 100 mg NO_3^- l^{-1} and even as high as 150 mg NO_3^- l^{-1}

Drinking water

The nitrate concentration in 1981 in the Netherlands is shown in Table 4.

Norway

85% of the population of about 4.2 millions is supplied from surface waters of very low nitrate concentrations (about 2 mg NO_3^- l^{-1}). The nitrate

concentration in private wells sometimes exceeds the Norwegian guideline value of 11 mg NO_3^- l^{-1} (Anon, 1983b), but most groundwater has concentrations below 9 mg NO_3^- l^{-1}.

Sweden

Approximately half of the drinking water is derived from surface water and half from groundwater. No problem exists with high nitrate levels in drinking water derived from surface water. However, a recent report (Thours and Joelsson, 1982) estimates that at least 100,000 people use drinking water from private wells with nitrate concentrations above 50 mg NO_3^- l^{-1}. In addition, public water supplies derived from groundwater in some areas in Southern Sweden have levels above 50 mg NO_3^- l^{-1}.

During the last 20 years, nitrate levels in drinking-water derived from groundwater have increased in some agricultural areas in Southern Sweden. Table 5 shows nitrate concentrations in drinking water in Sweden over the period 1968 to 1971. The maximum value found was 84 mg NO_3^- l^{-1} for Gotland.

Switzerland

About 98% of the population of 6,5 million receives drinking water with nitrate concentration less than 30 mg NO_3^- l^{-1}, and an average value of 10 mg NO_3^- l^{-1}. Surface water with low nitrate concentration serves about 27% of the population. However, nitrate levels in groundwater and well waters are increasing and have doubled since 1950 (Anon, 1979; Micain, 1980).

United Kingdom

Surface Waters

Figure 2 illustrates nitrate data for major UK rivers over periods of up to 34 years (UK, DOE, 1986).

Surface waters provide about 70% of the public water supplies in Britain. In much of Scotland and Wales where the nitrate concentrations are generally very low, surface waters provide over 90% of the supplies. In Central and South-Eastern England (where some river concentrations can exceed 50 mg NO_3^- l^{-1}) the proportion may be as low as 30%, although this

proportion may rise as water undertakers look to relatively low nitrate surface waters to blend with high nitrate groundwater.

Groundwaters

Groundwater provides approximately 30% of the public water supplies in Britain though the percentage varies a great deal from region to region. In parts of Southern and Eastern England the percentage may be as high as 70% whereas in Wales and Scotland it is less than 10%.

Nitrate concentrations have been increasing steadily in groundwaters. In 1970, 60 groundwater sources exceeded a nitrate concentration of 50 mg NO_3^- l^{-1}. By 1980, approximately 90 groundwater sources intermittently contained nitrate in excess of 50 mg NO_3^- l^{-1}, while by 1984, 104 sources were so affected. Some groundwater sources have been taken out of supply, and others blended with low nitrate surface waters. A great deal of research into the nitrate question is continuing in the UK (UK, DOE, 1986).

Drinking Water

Table 6 shows the nitrate levels in UK tap water for 1985 (UK, MAFF, 1987).

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TABLE 1

PERCENTAGE OF DRINKING WATER
DERIVED FROM GROUNDWATER SOURCES.

Country	% from groundwater
BELGIUM	76
DENMARK	99
FRANCE	68
FEDERAL REPUBLIC OF GERMANY	73
REPUBLIC OF IRELAND	20
ITALY	88
LUXEMBOURG	73
NETHERLANDS	65
SPAIN	20
PORTUGAL	(not available)
UNITED KINGDOM	32

TABLE 2

PERCENTAGE OF FRENCH POPULATION SERVED, AND RELEVANT DISTRIBUTION SYSTEMS AS A FUNCTION OF THE MEAN NITRATE CONCENTRATION DRINKING WATER (Anon, 1979-81).

Nitrate concentration mg NO_3^- l^{-1}	0-25	25-50	50-100	>100	Total
Population served %	80.44	17.38	2.12	0.06	100 53 million
Number of Distribution % Systems	83.75	13.32	2.78	0.15	100 (1974)

TABLE 3

PERCENTAGE OF GERMAN POPULATION AS A FUNCTION OF MEAN NITRATE CONCENTRATION IN PIPED DRINKING WATER (Aurand, 1985)

	Mean Nitrate Concentration (mg NO_3^- l^{-1})				
Distribution (%)	0-10	10-30	30-50	50-80	>80
N= (3387)	49.4	34.9	11.7	3.31	0.64

TABLE 4

NITRATE CONCENTRATION IN DRINKING WATER IN THE NETHERLANDS (1981).

Total number of pumping station : 257

Nitrate concentration (mg NO_3^- l^{-1})	0-10	10-25	25-50
Frequency (%) of population supplied	84.8	12.8	2.4

TABLE 5

NITRATE CONCENTRATIONS IN DRINKING WATER IN SWEDEN 1968 TO 1971 (Socialstyrelsen Redovisar, 1981).

Number of Tests = 4102

Nitrate concentration (mg NO_3^- l^{-1})	0-30	30-50	>50
Frequency (%) of population supplied	98.5	1.2	0.3

TABLE 6

NITRATE LEVELS (mg NO_3^- l^{-1}) IN UK TAP WATER (1985).

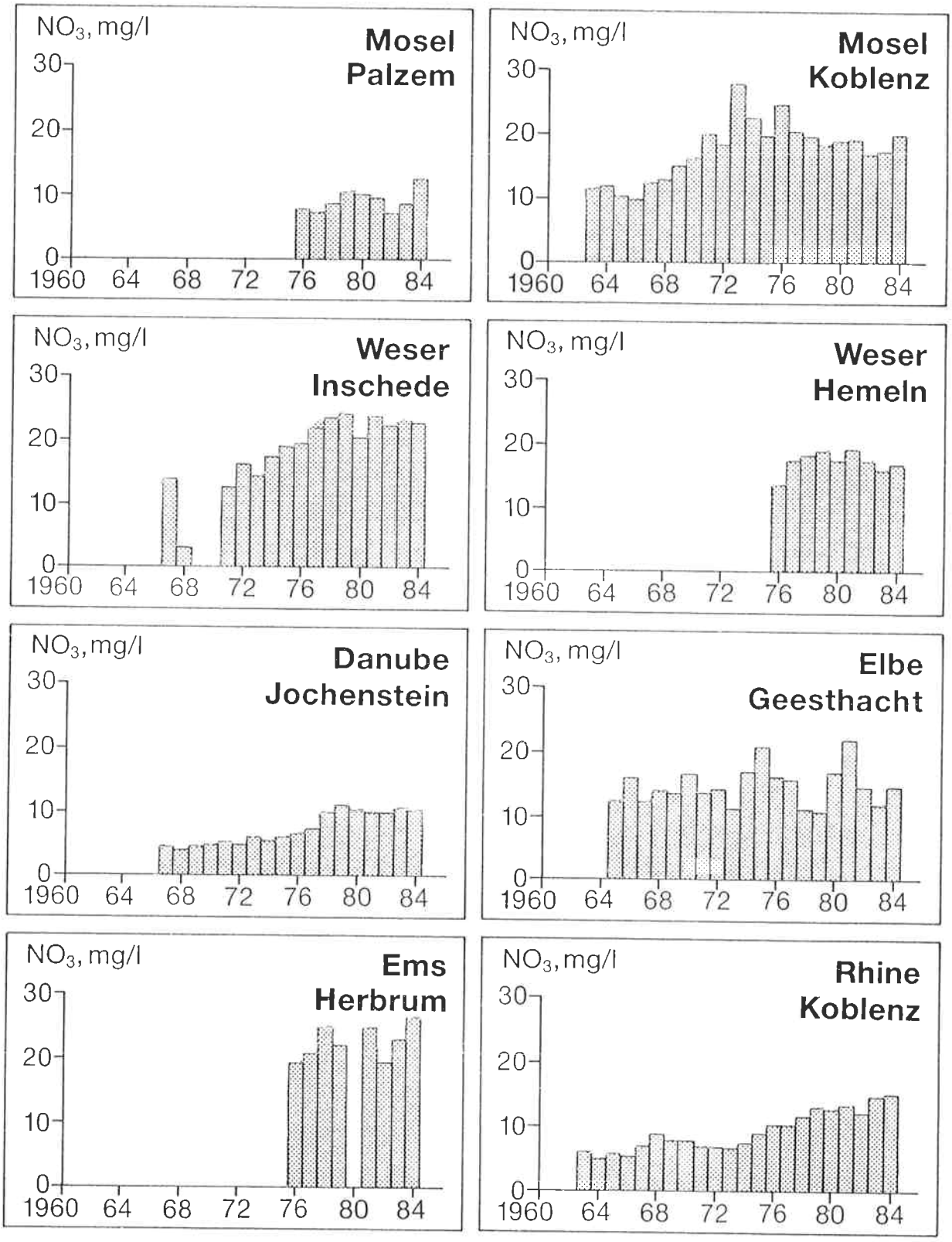
(UK, MAFF, 1987)

The data are for mean nitrate levels, in calendar year 1985, for public water supplies.

Nitrate levels	Population receiving supply %
0-9.99	41
10-19.99	16
20-29.99	24
30-39.99	16
40-49.99	2.0
50-59.99	1.0
60-69.99	0.053
70-79.99	0.065
80-89.99	0.0014*
>90	0

* 860 people

Nitrate content of selected surface waters (rivers) in the Federal Republic of Germany

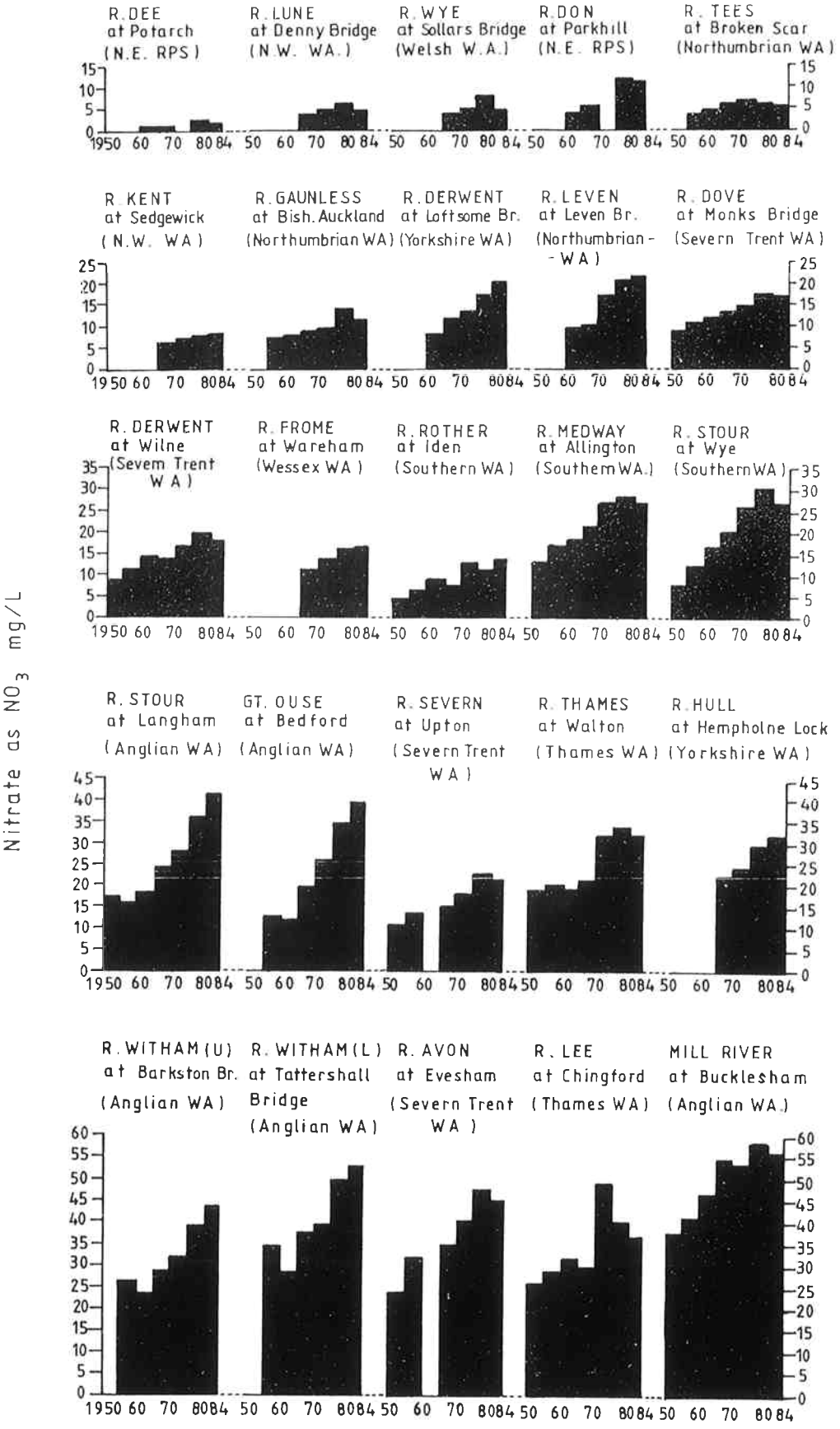


(After: Bundesamt für Gewässerkunde, Koblenz
published in Daten zur Umwelt 1986/87, Umweltbundesamt, Berlin)

FIGURE . 2 .

FIVE - YEAR MEAN NITRATE CONCENTRATIONS 1950 – 1980 TOGETHER WITH FOUR - YEAR MEAN NITRATE CONCENTRATION 1981 – 1984 .

NITRATE CONTENT OF MAJOR U.K. RIVERS .



APPENDIX 3

ANALYTICAL METHODS FOR DETERMINATION OF NITRATE AND NITRITE IN FOODS.

1. ANALYTICAL METHODS.

Before attempting to review the mass of data available relating to nitrate and nitrite in foods is important to establish if there are likely to be serious errors involved in the methods of analysis employed.

The topic was reviewed by the NAS (1981). Further progress has been achieved in analytical methodology since that time. The following briefly reiterates the findings of the above mentioned review and extends them in the light of subsequent knowledge.

1.1. NITRATE

Methods for nitrate analysis are based on

1. Nitration of phenolic compounds (AOAC, nitrate and nitrite in meat, 1980)

2. reduction of Nitrate to nitrite and determination as nitrate (AOAC, nitrate and nitrite in cheese, 1980).

3. direct measurement of absorption by nitrate in UV wavelengths.

4. by atomic absorption spectroscopy of the ion pair formed by nitrate with bis (2,9 dimethyl 9, 10 - phenanthroline) (Copper I) and extraction into 4 methyl - pentanone (MIBK) (Silva, Gallego and Carcel, 1986).

5. By specific ion electrode (Adamowicz, Ballino, Mars and Otto, 1986; Sherken, 1976; Mazoyer, 1972).

6. by HPLC/ion chromatography using detection of nitrate by UV spectroscopy. (Gerritse, 1979; Leuenberger et al, 1980; Iskandarani

and Pietrzyck 1982 (a, b); DeKleijn and Hoven, 1984; Jackson et al, 1984; Saitoh and Oikawa 1984; Wootton, et al, 1985; Eggers and Cattle, 1986)

7. by treatment with benzene and sulphuric acid and detection of the nitrobenzene formed by gas chromatography using a thermal energy analyser (Ross and Hotchkiss 1985)

(1-3 reviewed by Usher and Telling 1975).

1.1. NITRITE

Methods for nitrite are based on :

1. Diazotisation of a primary amine in acid solution and subsequent reaction with an aromatic compound to form an azo colour that is measured spectrophotometrically. (Griess Ilosway Reaction).

2. By acidification and measurement of nitric oxide by chemiluminescence. (Walters et al, 1978)

3. By HPLC/ion chromatography, with detection by UV, either as nitrite or after oxidation to nitrate. (Gerritse, 1979; Leuenberger et al, 1980; Iskandarani and Pietrzyck 1982 (a, b); DeKleijn and Hoven, 1984; Jackson et al, 1984; Saitoh and Oikawa 1984; Wootton, et al, 1985; Eggers and Cattle, 1986)

2. ANALYSIS OF FOODSTUFFS

Some of the methods described for analysis of nitrate and nitrite in various foods since 1970 are indicated in the table below.

	Water	Vegetable	Meat Products	Dairy Products
AOAC (1980)	+	+	+	+
Fudge & Truman (1973)	-	-	+	-
Sen and Donaldson (1978)	-	+	+	+
Gerritse (1979)	+	-	-	-
Luenberger et al (1980)	+	+	-	+
Iskandarani & Pietrzyk (1982 b)	+	+	-	-
Lox & Okabe (1982)	-	+	-	-
Hertz & Baltensburger (1984)	-	+	-	-
Saitoh & Oikawa (1984)	-	+	-	-
Jackson et al (1984)	-	+	+	+
De Kleijn & Hoven (1984)	-	+	+	-
Ross & Hotchkiss (1985)	-	-	-	+
Wooton et al (1985)	-	+	+	-
Egger & Cattle (1986)	-	-	+	-
Silva et al (1986)	-	+	+	-

Analysis for nitrate or nitrite in water is relatively easy compared to foodstuffs since no clean-up is needed. The methods used in a number of countries were briefly reviewed by WHO (1985).

All the methods of analysis of foodstuffs involve some extraction of the nitrate and nitrite into aqueous solution, followed by some form of clean up of the extract prior to analysis.

Methods for clean up are deproteinisation using potash alum, borax, or zinc acetate and potassium ferrocyanide (Carrez reagents), or by techniques such as gel infiltration or ion exchange.

The Carrez reagents appear to be the most frequently used method. It is important that the zinc acetate is added before the ferrocyanide to prevent destruction of the nitrite. The technique is widely used with vegetables and with meat products. As the final solution has a pH about

5.0 it is recommended the solution is made slightly alkaline by addition of sodium hydroxide. (Usher and Telling).

The AOAC methods for these analysis use zinc sulphate and sodium hydroxide to remove both proteins and fat followed by reduction of nitrate by cadmium column to nitrite for analysis by the Griess method (Nitrate + Nitrite). Nitrite is determined by omitting the reduction step. Nitrate is obtained by difference. In meat product analysis, the sample extract is cleared using phosphotungstic acid followed by silver sulphate/ammonium hydroxide solution. Nitrate is then analysed by the xylenol method (AOAC). For nitrite analysis the sample is simply extracted for 2 hours in water and filtered before colour development using the Griess reagents.

3. PRECISION OF METHODS

The detailed review by Usher and Telling (1975) shows that there can be problems in methods based on the reduction of nitrate to nitrite prior to spectrophotometric determination of the nitrite by the Griess-Ilosvay technique (it gives nitrate + nitrite). In these methods nitrite is determined by omitting the reduction step. Nitrate is obtained by difference. There are several potential problems. With vegetables interference due to ascorbic acid ; and with meat interference due to SO_4^{2-} or PO_4^{3-}, the "meat blank" and a dilution effect. It has also been shown by Sen and Donaldson that control of pH during extraction is important as up to 60% of nitrite present can be destroyed. Additionally it is also important to control pH during the colour development stage to maximise colour development (Davis et al, 1985). (This is not done in the AOAC method for cheese (nitrate and nitrite) or for meat (nitrite only).

Methods based on nitration using strong acids have been criticised because in the presence of organic material charring produces interference and high values. The AOAC method for analysis of meat products precipitates organic matter, avoids interference by steam distillation of the nitro-xylenol and will detect nitrate down to about 6 mg NO_3^-/kg. Below this level the sample extract has to be concentrated.

Fiddler and Fox (1978) studied sample preparation prior to analysis for nitrate by the reduction technique and concluded that the AOAC methods gave the highest yields of nitrite (3-300% greater than other methods) and were to be preferred.

The detection of nitrate by absorption in the UV region has been used for water analysis for many years. The various methods listed (under Nitrate methods, no. 6) use the range of techniques mentioned for clearing and deproteinising food samples, most frequently based on the use of the Carrez reagents. The various HPLC techniques described, then provide separation of the nitrate from the nitrite and permit simultaneous analysis. These newer methods can easily detect NO_3^- and NO_2^- at 1-10 ppm levels in solution which means analysis of NO_3^- and NO_2^- at ppm levels. Leuenburger et al (1980) quote a detection limit of 0.05 ppm to 10 ppm depending on the substrate analysed. The methods compared well with the nitroxylenol method using steam distillation for clean up. On salad crops HPLC/UV ranged 98% to 113% of the nitroxylenol method. For water samples comparison with the German official spectroscopic method gave results ranging 99.3 - 103.4% of the spectroscopic method. Saitoh and Oikawa (1984) describe a similar method using a different ion chromatography system for use with salted vegetables and Jackson et al (1984), describe a method using chloromethane sulphonate as eluent, which does not absorb in the UV region, and applied it to vegetables, cheese and cured meat. Where they found differences in the results compared with a spectrophotometric method they considered these to be due to failure of the spectrophotometric method, "poor sensitivity, high blank corrections, and the need to reduce nitrate to nitrite". Results range for nitrate 98.6 to 125% and for nitrite 103-143%* of the spectrophotometric value *(although the percentage spread in the latter case is of little significance when measuring 1.4 mg/kg).

By contrast Wootton et al (1985) show that the HPLC technique they have used gives low values for nitrite and an increase in nitrate when ascorbic acid is present (total nitrite plus nitrate remains constant). Presence of salt (NaCl) increased both nitrite and nitrate levels, nitrite levels by 10% and nitrate by 25% with 5 mg salt per litre of solution analysed by the spectroscopic method. Saitoh and Oikawa (1984)

describe a similar method using a different ion chromatography system for use with salted vegetables and claim detection limits of 5 ppb for NO_3^- and 10 ppb NO_2^-.

Silva et al (1986) have described an automated method using atomic absorption spectroscopy of a copper complex in organic solvent. This method claims several advantages, principally tolerance of both phosphate and ascorbate in the substrate though chloride and bromide interfere but this can be controlled. With additions of 1 mg/kg of nitrate plus nitrite recoveries ranged 95.6 to 102.8% with a mean of 100%. Relative standard deviations were 2%.

Methods based on the nitrate ion electrode suffer from the fact that the electrode is also sensitive to other ions, whilst these difficulties can be coped with the technique is not widely used.

4. CONCLUSION

1. Analysis of nitrate and nitrite in water presents few technical problems.

2. In contrast analysis of nitrate and nitrite in food presents substantial problems which need to be considered when assessing nitrate and nitrite intake from these sources.

It is clear from the literature that the long established methods based on reduction of nitrate to nitrite followed by colourimetric determination of the nitrite formed are liable to errors. The AOAC method for nitrate based on nitration of xylenol is more robust, even so its limit of detection is about 6 mgs/kg. Where nitrate or nitrite levels are low (less than 10 mg/kg) these methods are not to be recommended. At low concentrations newer methods based on HPLC and UV detection have advantages though attention must still be paid to the details of sample preparation, extraction and purification for final assay.

The method of Silva et al (1986) based on atomic absorption spectrometry also appears to offer some advantage in terms of sensitivity and precision.

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