N-NITROSAMINES AS POTENTIAL FOOD CARCINOGENS

by

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Introduction

That dimethylnitrosamine is acutely hepatotoxic to a number of laboratory animals was first demonstrated by Barnes & Magee[1] in 1954. They subsequently showed[2] that it is carcinogenic to rats, feeding a diet containing 50 parts per million of dimethyl nitrosamine produced malignant liver tumours in nearly all the animals in less than a year. Subsequent studies have revealed that many N-nitrosamines are carcinogenic to a wide range of experimental animals at low doses, in some cases as low as a few parts per million. Although carcinogenicity to man has not been formally demonstrated, the evidence available to date suggests that it would be imprudent to dismiss this possibility. Furthermore, since the principal route in the formation of N-nitrosamines is the reaction, in slightly acid solution, of secondary amines with nitrite there is an *a priori* case for the formation of N-nitrosamines in protein-containing foods preserved with sodium nitrite. Consequently a potential risk to man due to N-nitrosamines from this source and possibly other environmental sources must be recognised. Many studies have been reported, and others are in progress, aimed at providing the evidence upon which the extent of this hazard can be assessed. In this paper the principal biological, biochemical and chemical evidence so far accumulated is summarised and correlated to produce a rationale of the nitrosamine problem.

1 Animal Studies

The most extensive studies using experimental animals have been conducted by Druckrey and his co-workers (for a summary of their findings and those of other workers see Magee & Barnes[3]). Over 80 N-nitrosocompounds have been studied and the majority of them have induced lesions in the animals. Of particular interest is the ability of some of the more active compounds to induce cancer or other toxic effects after only a single dose[4,5]. For example dimethynitrosamine in doses of 25 mg/kg body weight produced liver necrosis in rats whether administered orally, intravenously, interpentoneally or subcutaneously. The number of organs in which tumours have been induced by N-nitroso compounds is large and in some instances the compounds exhibit marked organ specificity, thus dibutylnitrosamine induces bladder cancer in the rat[4].

Of relevance to possible carcinogenic effects in man are the life-time feeding studies with experimental animals using relatively low dosages. Table I, abstracted from ref 3, lists those nitrosamines which have given positive results by this means and the principal organs attacked.

Table I

Carcinogenic Properties of some N-nitrosamines:
Life Span Studies

Compound	Animal Species	Organ Affected
Dimethylnitrosamine	Rat	Liver
	Mouse	Liver
	Hamster	Liver
Diethylnitrosamine	Rat	Liver
	Rat	Aesophagus
	Mouse	Liver
Di-n-propylnitrosamine	Rat	Liver
Di-n-butylnitrosamine	Rat	Bladder Aesophagus
Methylbenzylnitrosamine	Rat	Aesophagus
Ethylisopropylnitrosamine	Rat	Liver Aesophagus
Ethyl-n-butylnitrosamine	Rat	Liver Aesophagus
	Mouse	Forestomach
N-nitrosomorpholine	Rat	Liver
	Mouse	Liver
N-nitrosopiperidine	Rat	Aesophagus
	Rat	Liver
N-nitrososarcosine	Rat	Aesophagus
N-nitrososarcosine ethyl ester	Rat	Forestomach tongue

It is evident that N-nitroso compounds are extremely potent carcinogens with respect to animals, a wide range of animal species has proved susceptible[5] and since this includes the monkey[6] it must be considered likely that man may be also. Animal studies performed to date do not allow a conclusion as to whether a dose-response relationship exists in N-nitrosamine carcinogenicity.

Biochemical Studies

Elucidation of the biochemical mechanisms of nitrosamine carcinogenicity is clearly of fundamental importance. Studies with rat liver slices[7] have shown that the organ is capable of metabolising dimethylnitrosamine. Other organs, with the exception of the kidney and lung are metabolically inactive. It has been established, using dimethyl-and other nitrosamines labelled with carbon -14 in the alkyl groups, that nucleic acids and proteins of the animal liver become alkylated.[8-10] With the nucleic acids the alkylation occurs mainly at the 7- position of guanine; adenine and cytosine are methylated to a lesser extent as demonstrated by experiments using ^{14}C- dimethylnitrosamine.[11] Specific activity measurements indicate that the methyl group is transferred direct from the nitrosamine to the nucleic acid without intermediate dilution[7]. These studies suggest that the carcinogenic activity of the dialkylnitrosamines involves metabolic conversion in the organ and the subsequent formation of an intermediate compound which is biologically active. Whether this hypothesis applies for heterocyclic nitrosamines is currently uncertain but nitrosomorpholine induce liver cancer and it undergoes metabolism in the liver with limited alkylation of RNA[12]. Earlier suggestions that the biologically active alkylating agent is a diazoalkane[3,13,14] lose support in the light of the findings of Lijinsky, Loo and Ross[15] who extracted nucleic acids from rats treated with fully deuterated dimethylnitrosamine. The molecular weight of the 7-methyl guanine showed that the methylating agent was $-CD_3$. The intermediate formation of diazomethane would require the methylating agent to be $-CD_2H$. Although alkylation appears the most likely process giving rise to biological activity of nitrosamines it is by no means fully established, other reactive intermediates such as nitrous acid, hydrazines and aldehydes may possibly be involved.

Montesano and Magee[16] have demonstrated that excised human liver slices metabolise dimethylnitrosamine at a rate similar to that by rat liver and that the extent of nucleic acid methylation is also comparable. This would suggest that man and the rat have have similar levels of sensitivity to the carcinogenic action of dimethyl nitrosamine.

3. Formation of N-nitrosamines

The principal mode of formation of N-nitrosamines is the reaction between secondary amines and nitrous acid,

$$\begin{matrix} R_1 \\ R_2 \end{matrix} \!\!> NH + HNO_2 \rightarrow \begin{matrix} R_1 \\ R_2 \end{matrix} \!\!> N - NO + H_2O$$

However, Fiddler et al have demonstrated[17] that dimethylnitrosamine is formed on reacting nitrous acid with several tertiary and quaternary amines containing methyl groups, albeit usually in much lower yield than from secondary amines. The highest

yields from these sources, about 50% of that from secondary amines, were obtained from 2-dimethylaminoethyl acetate and the methyl ester of N,N[1] dimethylglycine. An earlier claim[18] that N-nitrosamines are formed as by-products of the Maillard reaction (amino acids + glucose) has subsequently been disproved[19], Inadequate specificity of the analytical methods initially used was responsible for the unsupported findings.

In seeking to assess the potential hazard to man in terms of possible dietary or environmental intake of N-nitrosamines it is important to fully understand the rate and conditions under which the secondary amine - nitrite reation occurs. Although the reaction proceeds quite rapidly when high concentrations of reactants are used it is slow and therefore markedly temperature dependent at concentrations approaching those likely to be encountered environmentally or in food. The kinetics of the reaction have been examined in detail by Mirvish[20,21]. The reaction is described by the following equations

$$\text{rate} = k_1 \left[R_2NH\right] \left[HNO_2\right]^2 \text{ - - - - - - - - - - - - - - - (1)}$$

or

$$\text{rate} = k_2 \left[\text{total amine}\right] \left[\text{total nitrite}\right]^2 \text{ - - - - (2)}$$

in which k_1 is independent of pH but k_2 is pH dependent. Mirvish's data[21] for several secondary amines and amino acids are given in Table II.

TABLE II

Rate constants for the nitrosation of some secondary amines and amino-acids

Amine	pK_a	Optimum pH	k_2 *	$k_1 \times 10^{-6}$
piperidine	11.2	3.0	0.027	8.6
dimethylamine	10.72	3.4	0.10	8.9
morpholine	8.7	3.0	14.8	15.0
mononitroso-piperazine	6.8	3.0	400	5.0
piperazine	5.57	3.0	5000	3.7
L-proline	-	2.25	2.9	-
L-hydroxproline	-	2.25	23.0	-
sarcosine	-	2.5	13.6	-

* Values at the optimum pH, in moles^{-2} $_1$ ^{2} min^{-1}.

Fig 1 depicts the pH dependence of the reaction, the maximum rate is at about pH 3.4 for the more basic amines and shifts to slightly more acid values as the amine basicity decreases. k_2 also increases with decreasing amine basicity. It should be noted from Fig 1 that even at the relatively high concentrations used theextent of reaction is far from complete even after 3 hours. In the author's Laboratory it has been shown that the reaction between 100 ppm diethylamine and 1000 ppm nitrite is incomplete after several hours at 98°C at the optimum pH. At room temperature little reaction occurred over a 24 hour period. These experiments were carried out in solutions bufferred with acetate because weak acid anions are known to catalyse the reaction, so the rates should be maximal.

Extrapolation from the results obtained on model systems to attempt the prediction of what might occur in biological situations, such as foods in which nitrite and secondary amines can co-exist, requires extreme caution, but it seems likely that the maximum risk attends thermally processed commodities bufferred in the pH range 3 to 4, an extremely rare combination.

N-nitrosamines are, in general, quite thermally stable. Fan and Tannenbaum[22] studied the decomposition at 110°C of dimethylnitrosamine (DMN) N-nitrososarcosine (N Sar) N-nitrosoproline (NPr) and N-nitrosopyrrolidine(NPy) as a function of pH. Table III summarises the results in terms of half-lives at various pH values. De-nitrosation appears to be a primary decomposition mechanism, in addition N-nitrososarcosine and N-nitrosoproline decarboxylate at low pH. The four compounds studied represent examples of several generic nitrosamine structuresand the results should enable partial prediction as to the thermal stability of other N-nitrosamines.

TABLE III

pH	Half-life, days			
	N Py	DMN	N Sar	N Pr
2.2	150	150	0.3	0.08
4.0	55	55	1.7	0.4
5.5	28	16	18	6.0
7.0	28	24	25	4.5
8.5	11.4	15	67	5.3
11.0	1.1	150	120	3.0
12.2	1.0	67	120	1.5

4. Nitrosamine Precursors

It is evident from Section 1 above that many N-nitrosamines possess carcinogenic activity but in order to formulate a judgement on the risk to man it is necessary to have an understanding of which of these may occur in materials likely to be ingested. This requires a knowledge of the identity and concentration of N-nitrosamine precursors in the environment.

Evidence regarding the nature and level of occurrence of the amine precursors, whether secondary, tertiary or quaternary, is meagre, and this is an area requiring much research before the extent of the N-nitrosamine problem can be properly circumscribed. The methylamines are known to occur in fish; it was a nitrite-treated fish-meal feeding stock which caused the deaths of sheep, dimethylnitrosamine being identified as the hepatotoxic agent.[23,24] There are many compounds containing the -NH group which occur in food or in vivo; these include proteins, nucleic acids, proline, pyrrolidine, tryptophan, histidine, arginine, lysine, piperidine, tyrosine and certain polyamines. Some of them are known to be nitrosatable, for others the necessary research has yet to be undertaken.

A number of widely used drugs contain secondary amino groups and Lijinsky, Conrad and Van de Bogart [25] have demonstrated that some react readily with nitrite in the pH range 2.0 to 4.4 to yield nitrosamines. For example oxytetracycline and aminopyrine give high yields of dimethylnitrosamine and disulfiram and nikethamide give low yields of diethylnitrosamine. A 40% yield of nitrosophenmetrazine resulted within 3 hours when nitrite and phenmetrazine were reacted in vivo in rabbits and rats by incubation in the tied-off stomach. Since drugs can be administered in appreciable quantities they may well represent a potential source of in-vivo N-nitrosamine formation in patients.

The nitrite necessary for N-nitrosamine formation can arise in two ways, the use of sodium nitrite as a preservation for certain foods (cured meats and some cheese in the U.K., certain marine fish in the U.S.A.) and from nitrate by reduction with micro-organisms. The use of sodium nitrite as a food additive is governed by national legislation which specifies both the foods in which it can be used and the permitted quantities. Nitrate ion occurs widely in the environment, most drinking waters contain 10 to 50 ppm and it is a fairly common constituent of vegetables, and of course the soil, through fertilizer usage. Nitrate can be reduced to nitrite in vivo and one must therefore consider not only the direct ingestion of nitrosamines but the possibility of their formation in vivo through ingestion of nitrate, subsequent reduction to nitrite and reaction with amine constituents of foods, beverages or drugs. Sander[26-28] has demonstrated the en vivo formation of nitrosamines by feeding nitrite together with amines. The non-carcinogens N-nitrosodiphenylamine was detected in the stomach when diphenylamine and nitrite were fed to rats. Tumour formation occurred when morpholine and methylbenzylamine were fed together with nitrite. Similar effects were

were observed when the nitrite was replaced by potassium nitrate together with a nutrient medium, thus demonstrating the in-vivo reduction of nitrate to nitrite. It was further noted, in agreement with results of in-vitro studies that the more highly basic amines are much less readily nitrosated, in experiments where dimethylamine was fed the formation of dimethylnitrosamine was negligible. The reactant concentrations used in these experiments were higher than those likely to be encountered in the human situation. Indeed the ready absorption of nitrite after administration renders experiments at lower concentrations difficult.

The bacterial production of nitrosamines observed by Sander et al in the above experiments on rats cannot be directly extrapolated to the human body, bacterial reduction is far more likely in rats because the rat stomach and upper small intestine are heavily contaminated dur tothe lower acidity conditions. However the fact that the bacterial reduction in Sander's experiments occurred at higher pH values (5 to 6) than those at which chemical nitrosation occurs led Hawksworth & Hill[29,30] to consider in detail which sites in the human body could have amine, nitrate and bacterial concentrations sufficient to produce nitrosamines. The two main possibilities appear to be the gut, where lethicin is bacterially degraded to choline and N-dealkylated to give dimethylamine, and the urine of people with urinary tract infections. The latter ar mostly due to E coli which can produce nitrosamines in the bladder of the rat. Pyrrolidine and piperidine, both of which are present in human urine, were nitrosated in this way and also dimethylamine, albeit in extremely low yield. Further studies by Hill and Hawksworth reveal that the bacterial nitrosation reaction is complex, not all strains possessing nitrate reductase are active and some non-nitrate-reducing species are, for example certain lactobacilli, clostridia and group D streptococci.

Tannenbaum[31] has demonstrated the presence of small, (less than 10 ppm) relatively constant concentrations of nitrite in human saliva. This would seem to be the result of bacterial reduction of nitrate in the mouth. It represents a possible nitrosating source, more particularly if thiocyanate, which catalyses nitrosation, is present. It has been reported that the highest thiocyanate concentrations are found in the saliva of smokers[32] and pregnant women.

5. Analytical Studies

The past two or three years have seen rapid advances in the development of methods for determining N-nitrosamines and some can be determined to a sensitivity of a few µg/kg To the analytical chemist the problem falls into two distinct parts depending upon the physical properties of the nitrosamines. Proven methods so far developed utilise distillation from steam or partial vacuum and consequently the current analytical capability is limited to those N-nitrosamines which are volatile under these conditions. These comprise the lower alkylnitrosamines and a few nitroso derivatives of heterocyclic compounds. As yet there are no suitable methods for non-volatile nitrosamines and until these are available it will not be possible to assess the full extent of the the nitrosamine hazard in practical terms.

Food surveys for steam-volatile nitrosamines have been undertaken in Canada, the USA and UK and further work is in progress. Already there is a considerable measure of agreement between results reported from the three sources. Initially surveys have been predominantly directed at those foods where sodium nitrite is permitted as an additive. Table IV summarises the results of the first survey carried out at the author's laboratory in conjunction with the Ministry of Agriculture, Fisheries and Food.

TABLE IV

UK N-Nitrosamine Survey Results

Commodity	Number of Samples Examined	Number of Positive Results in Concentration Ranges (µg/kg)								
		DMN			DEN	N-PYR				N-PIP
		<1	1-4	5-9	<1	<1	1-4	5-9	10-40	<1
Fried Bacon	24	7	6		1	4	6	1	1	1
Fish										
Fresh	13		3							
Fried	10		7	2						
Cheese	11		6							
Salami	6		1							

DMN = Dimethylnitrosamine
DEN = Diethylnitrosamine
N-PYR = N-nitrosopyrrolidine
N-PIP = N-nitrosopiperidine

Dimethylnitrosamine and N-nitrosopyrrolidine were the only species regularly detected and this accords with USA and Canadian experience. The levels found are also broadly similar.

6 Further Research

Although published results on the N-nitrosamine content of foods are limited in number at present it must be accepted that volatile N-nitrosamines are frequently present in some foods at concentrations of a few µg/kg. Work is now required in the following areas.

5.1 Further animal studies to assess whether a dose-response relationship exists and whether there is a no-effect level. Such studies must endeavour to ascertain whether the low concentrations of N-nitrosamines found represent a hazard. Animal feeding studies at these low levels are extremely exacting, not only are large numbers of animals required but practical problems, in particular cross-contamination and adventitious contamination, are formidable.

5.2 The development of methods for characterising and determining non-steam-volatile nitrosamines. Only when adequate methods come available can one ascertain which amine precursors are nitrosatable. Such information would then point the way to further animal studies.

5.3 Means of eliminating or limiting N-nitrosamine formation in food during nitrite treatment. In this connection Mirvish et al[34] have recently shown that ascorbic acid markedly reduces the extent of nitrosation of morpholine, piperazine, N-methylaniline and oxytetracycline, presumably by competition for nitrite. The treatment was, however, only partially successful for dimethylamine. Whether a nitrite-ascorbate preservation method has any potential in food processing must now be examined. Ascorbic acid is an acceptable food additive and is a common constituent of processed meats.

5.4 Alternatives to sodium nitrite. Research is already proceeding actively on these lines. Elimination of sodium nitrite would remove a major nitrosamine precursor but it would drastically modify the ham and bacon industry and may be premature in the absence of proof that ambient levels of N-nitrosamines are toxic to man.

5.5 Epidemiological studies. These represent perhaps the only method of demonstrating whether or not N-nitrosamines are carcinogenic to man. The mounting of such studies is extremely difficult but several possibilities are under consideration. In particular the International Agency for Research on Cancer is initiating a study in Iran.

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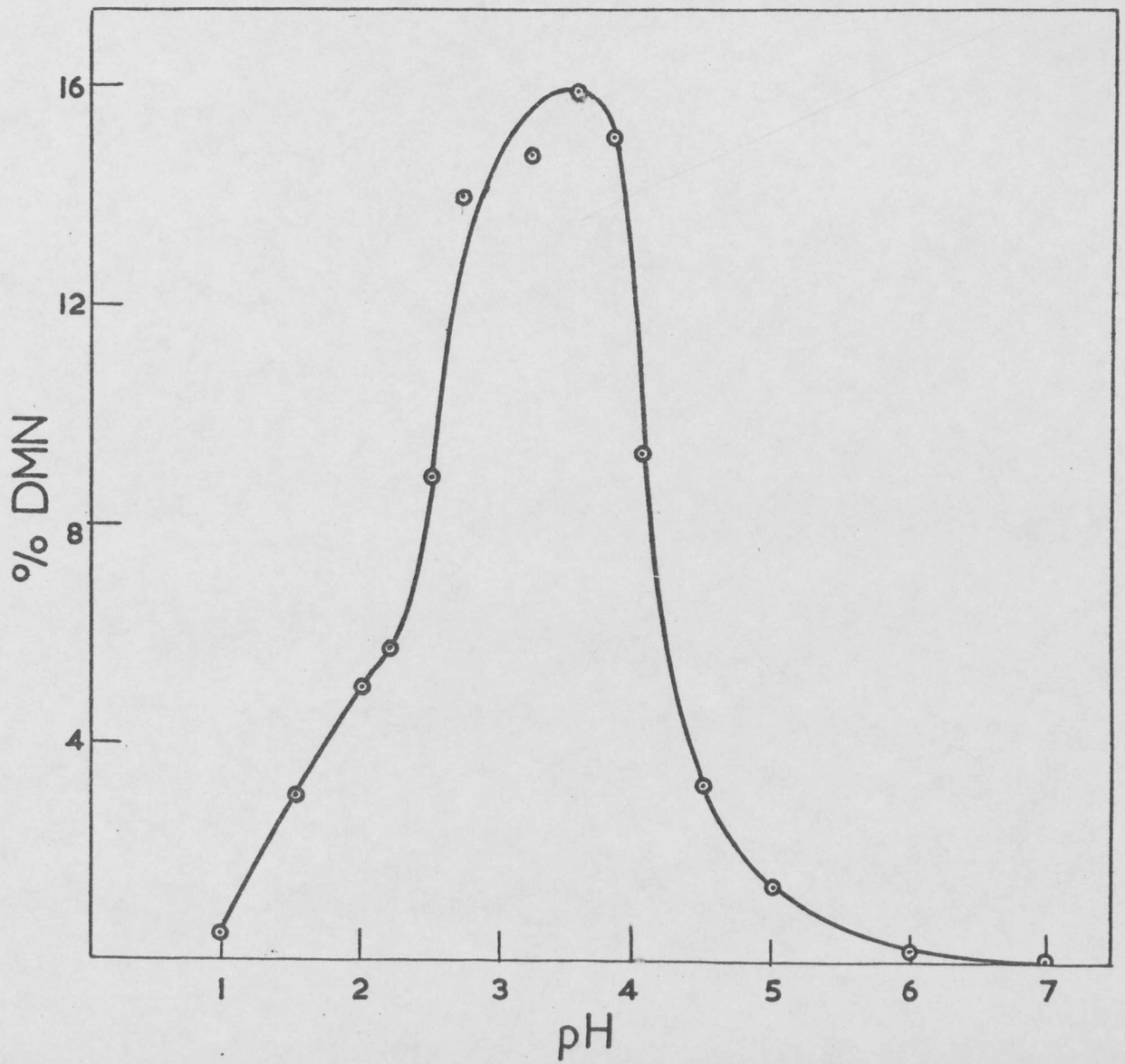
Fig 1



Dependence of DMN formation on pH. Standard reaction mixtures containing $Me_2NH.HCl$ (0.02 m), $NaNO_2$ (0.1 m), NaCl (0.05 m) and various buffers were reacted for 3 hours at 25°