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Commentary

Relevance of nitrosamines to human cancer

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Introduction

It is now about three decades since the hepatocarcinogenic effect of N-nitrosodimethylamine (NDMA)* was demonstrated in rats (1) and the suggestion was made that N-nitrosamines can be formed following nitrosation of various amines (2). Direct proof that such nitrosation reactions can occur was provided by Ender *et al.* (3) who identified NDMA in nitrite preserved fish-meal, and by Sander and Seif (4) who demonstrated the formation *in vivo* of a nitrosamine in the acidic conditions of the human stomach. Since then, because of the potent carcinogenicity, wide environmental occurrence and easy formation of nitrosamines, considerable efforts have gone into determining their levels in the external and internal human environment, and attempts have been made to assess exposure and to correlate it with human cancer at specific sites. Fundamental research into molecular and cellular mechanisms of carcinogenesis has also concentrated on N-nitroso compounds (NOC), to find out why and how this class of carcinogens produces tumours in up to 40 animal species (5,6) and displays remarkable species, organ and cell specificity (7–11) and a wide variety of genetic effects (12,13).

We summarize briefly recent progress made in these research areas. Because of space limitations, the literature citations are not exhaustive and review articles are often referred to.

Human exposure to NOC

The introduction of the chemiluminescence detector (Thermal Energy Analyzer) by Fine *et al.* (14) represented a breakthrough in nitrosamine analysis and made it possible to assess human exposure to (mostly) volatile nitrosamines. Today, reliable methods for the analysis of a wide range of non-volatile NOC are still not available, although progress has been made (15–18).

The occurrence of NOC in several matrices and the resulting potential human exposure have been tabulated (10,19,20 and Tables I–III). Such can be divided into endogenous exposure, though formation of NOC *in vivo* from precursor amines and nitrosating agents (Table I), and intake of preformed NOC from occupational and environmental sources (Tables II and III). Because of recent developments in detection methods, in the following we give some emphasis to endogenously formed NOC. This shall, however, not detract from the fact that human exposure to performed NOC from a variety of sources is firmly established.

In vivo formation of NOC

Quantitation of NOC formed *in vivo* in humans became feasible only recently due to lack of non-invasive methods (31), although their endogenous formation from ingested precursors had been suggested to be the largest single source of exposure for the general population (32). Both nitrosatable amino compounds and nitrate (referred to below as precursors) are commonly present in foodstuffs and prescribed drugs, and nitrate is easily reduced to nitrite by bacteria in the saliva or in the achlorhydric stomach. Using a newly-developed method for assessing endogenous nitrosation (see below) it has now been established unequivocally that NOC are formed in the human body, even after intake of levels of precursors that are considered to be normal daily amounts (see Table I). Therefore, increasing attention has been paid to the detection of NOC formed *in vivo*, which add to the body burden of carcinogens derived from exogenous intake, as suggested previously by Sander (33).

The nitrosoproline (NPRO) test, which is a simple, sensitive non-invasive method for the quantitative estimation of endogenous nitrosation in man (31,34,35) makes it possible to examine the formation of NOC in human subjects *in vivo*. In this test, subjects are (i) given a vegetable juice containing nitrate, (ii) given, 30 min later, a proline solution, (iii) fasted for 2 h; (iv) asked to provide 24-h urine samples, which are analysed for nitrosated amino acids by gas chromatography, using a NOC-specific detector (the Thermal Energy Analyzer). Application of the NPRO test to humans involves no risk to their health, since there is substantial evidence that NPRO is not carcinogenic or mutagenic and that 90% is excreted unmetabolized (36,37). In contrast, concomitantly formed (carcinogenic) nitrosamines are not readily detectable, because they are formed in small amounts and/or they undergo rapid metabolism or reaction with cellular material. Thus, the amount of urinary NPRO (and of some other nitrosated amino acids) excreted/24 h per person following ingestion of proline and/or nitrate seems to be a valid index of endogenous nitrosation occurring in the mammalian body.

Studies in humans indicate that the amount of NPRO formed *in vivo* is proportional to the dose of proline administered but increases exponentially with nitrate intake (particularly with >260 mg/day per person). About 20 μg NPRO per 24 h were formed, i.e., yields of 0.002% and 0.004% of ingested nitrate (325 mg) and proline (500 mg), respectively (31), although large interindividual variations have been found in the several hundred subjects investigated so far (Table I). The yield of nitrosamine following nitrosation of a secondary amine is very much dependent on the basicity of the amino group (pK value); therefore, the rate of nitrosation of amines may be up to four orders of magnitude

*Abbreviations: NOC, N-nitroso compounds; NDMA, N-nitrosodimethylamine; NDEA, N-nitrosodiethylamine; TSNA, tobacco specific nitrosamines; NNN, N-nitrosonornicotine; NNK, 4-(N'-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; NAB, N'-nitrosoanabasine; NAT, N'-nitrosoanatabine; NPRO, N-nitrosoproline; NSAR, N-nitrososarcosine; NTCA, N-nitrosothiazolidine 4-carboxylic acid; NMTCA, N-nitroso-2-methylthiazolidine 4-carboxylic acid; NDElA, N-nitrosodiethanolamine; NPIP, N-nitrosopiperazine; NMOR, N-nitrosomorpholine; NDPhA, N-nitrosodiphenylamine; NMBzA, N-nitrosomethylbenzylamine; CAG, chronic atrophic gastritis.

Table I. Estimated amounts of NOC formed in humans *in vivo*

Study subjects/population (n = number)	(Reference)	Intake of precursors (dose)/modifying agents		NOC identified in urine[a]	Detected concentration μg/24 h urine	Estimated amount of endogenous NOC[b] (μg/day/person)
Healthy, non-smoking subject (n = 1, 1 month of observation)	(21)	Uncontrolled Western diet without (−) or with (+) ascorbate (3 x 100 mg/day for 15 days)	(−)	NPRO	3.4 (1.7−5)	~1
			(+)		2.5	
			(−)	NTCA	12 (1.8−49)	~10
			(+)		2	
			(−)	NMTCA	10 (3−16)	~5.5
			(+)		4.5	
			(±)	NSAR	<5	<2
Healthy subjects (n = 25)	(unpublished data)	nitrate rich meal and proline (500 mg)		NPRO NTCA NMTCA	14.8 (1.5−40) 2.4 (0−6.8) 2.1 (0−5.7)	~9
Volunteers (n = 3)	(22)	single p.o. dose of amidopyrine (500 mg) + nitrate + ethanol (20 g, inhibitor of NDMA metabolism)		NDMA	0.5−10 (8 h urine)	25−1000
(n = 4)	(23)	single p.o. dose of piperazine (480 mg) (fasted stomach)		NPIP	0.8−2.5	30−66
Smoking and non-smoking subjects (n = 21)	(21)	uncontrolled Western diet	smoker	NTCA	14 (3−25)	~8 (excess in smokers)
			non-smoker		6 (1−21)	
			smoker	NMTCA	7 (1−19)	~4 (excess in smokers)
			non-smoker		3 (0.4−11)	
(n = 28)	(24)	control diet and proline	smoker	NPRO	11.8	~8 (excess in smokers)
			non-smoker		3.6	
(n = 28)	(25)	single p.o. dose of nitrate (260 mg) + proline (500 mg)	smoker	NPRO	42	~25 (excess in smokers)
			non-smoker		17	
Subjects with various degrees of histologically proven chronic atrophic gastritis (n = 49)	(26)	single p.o. dose of nitrate (260 mg) and proline (500 mg)		NPRO	traces − 120	<120
Inhabitants of high risk (n = 142)		local diet			8.3 (0−30)	~9 (mean NPRO)
and		local diet + proline (3 x 100 mg)		NPRO	12 (1−67)	
		local diet + proline + ascorbate (3 x 100 mg)			3.5 (0−32)	
low risk (n = 96)		local diet		NPRO	3 (0−14)	<3 (mean NPRO)
areas for oesophageal cancer in N. China	(27)	local diet + proline (3 x 100 mg)			6 (0−40)	

[a]NPRO, N-nitrosoproline; NTCA, N-nitrosothiazolidine 4-carboxylic acid; NMTCA, N-nitroso-2-methylthiazolidine 4-carboxylic acid; NSAR, N-nitrososarcosine; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperazine.

[b]Because many of the NOC measured are excreted in the urine, exposure to other (carcinogenic) NOC will depend partly on the concentration and properties of amines available for nitrosation; depending on their pk value the yield of nitrosamines could be up to 4 orders of magnitude higher as compared to NPRO (28).

faster than that of proline (28). A kinetic model was formulated (38) on the basis of published data on the nitrosation kinetics of secondary amines and the carcinogenic potency of the resulting nitrosamines. This model makes it possible to determine the daily doses of amine and nitrite that induce tumours in 50% of rats after two-years' feeding and is potentially applicable to humans, if it is assumed that 1−10% of a nitrate dose is reduced to nitrite. The model indicates that the carcinogenic risk resulting from life-time exposure to endogenously formed nitrosamines may not be negligible when easily nitrosatable amino compounds (like aminopyrine) and nitrate are ingested, yielding nitrosamines that are potent animal carcinogens (39).

When the NPRO test was applied recently in clinical and field studies, several new sulphur-containing N-nitrosamino acid analogues were identified − N-nitrosothiazolidine-carboxylic acid (NTCA) and *trans* and *cis* isomers of N-nitroso-2-methylthiazolidine 4-carboxylic acid (NMTCA) (21,40−43). The daily excretion of these compounds (in one non-smoking human subject) varied over one month, but a diet supplemented with ascorbic acid significantly decreased the time averaged mean of total urinary nitrosamino acids (21,44) (Table I). NTCA and NMTCA present in human urine may therefore result from (i) intake of preformed NOC, (ii) intake of the respective parent amino precursors (thiazolidine 4-carboxylic acid and its 2-methyl derivative) and subsequent nitrosation *in vivo*, or (iii) an endogenous two-step synthesis (Figure 1) involving reaction of L-cysteine with the respective aldehyde (formaldehyde or acetaldehyde) followed by nitrosation. Thus, measurement of endogenously-formed NTCA and NMTCA together with NPRO in urine may provide an index of exposure of human subjects to nitrosamines or their precursors, e.g., nitrosating agents, certain aldehydes or aldehyde-generating compounds (21,37).

Inhibitors and catalysts of endogenous nitrosation

A number of inhibitors of N-nitrosation have now been char-

Table II. Occupational exposure to preformed NOC[a]

Source of exposure	Major nitrosamines identified[b]	Detected concentration range	Estimated exposure (μg/day/person)
Leather tanneries	NDMA,NMOR	0.05 – 47 μg/m³	20 – 180
Rubber and tyre industries	NMOR,NDMA	< 250	
	NDPhA	0.01 – 1230 μg/m³	15 – 150
	NPYR,NDEA		
Metal working industries	NDElA	8 – 600 mg/kg (grinding fluid)	> 50
Chemical industries			
Rocket fuel	NDMA		10 – 50
Dye manufacture	NDMA,NDEA	0.03 – 0.1 μg/m³	< 5
Surfactant production	NDMA	0.03 – 0.8 μg/m³	< 5
Foundries	NDMA,NDEA	0.024 – 1.4 μg/m³	> 5
Fish processing (fishmeal) industries	NDMA	0.01 – 0.06 μg/m³	< 5

[a]Collated from references 10,20,29.
[b]NMOR, N-nitrosomorpholine; NDPhA, N-nitrosodiphenylamine; NPYR, N-nitrosopyrrolidine; NDEA, N-nitrosodiethylamine; NDElA, N-nitrosodiethanolamine.

Table III. Environmental exposure of humans to preformed NOC[a]

Source of exposure	Major nitrosamines identified[b]	Detected concentration range	Estimated intake/exposure (μg/day/person)
Food	NDMA	variable, depending	0.5 – 1.2
	NPYR	on food item	0.1 – 0.2
Cosmetics	NDElA	1 – 48 000 μg/kg	0.4
Drugs and pesticides	NDMA	1 – 330 μg/kg (aminopyrine)	?
Household commodities			
Surfactants	volatile	46 – 150 μg/kg	?
Rubber products		1 – 280 μg/kg	
Air pollution -indoor	NDMA	10 – 130 μg/m³ (cig. smoke)	?
-outdoor		< 1 mg/m³	
Cigarette smoke (main stream)	NNN	0.2 – 3.7	~17
	NNK	0.1 – 0.44	
	NAB	0.15 ng/cig	
	NAT and others	0.14 – 4.6	
Snuff	NNN	0.8 – 33	~200
	NNK	0.2 – 4.6 mg/kg	
	NAB	0.01 – 1.9	
	NAT	0.2 – 40	
	NDElA	0.3 – 3.3 mg/kg	

[a]Collated from references 10,19,20,30.
[b]NNN, N-nitrosonornicotine; NNK, 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; NAB, N'-nitrosoanabasine; NAT, N'-nitrosoanatabine.

acterized that could be used in lowering exposure of humans to NOC (45). For example, intake of ascorbic acid or of orange juice strongly inhibits *in vivo* nitrosation (Table I); vitamin E was less effective (31,35). Polyphenols, which occur as mixtures in large quantities in the human diet (e.g., in vegetables, fruits, beer, soft drinks), were shown to inhibit nitrosation *in vivo* (35,46,47). Catalysts of N-nitrosation have also been identified, and exposure to NOC could be reduced by diminishing their impact (45). Cigarette smokers tend to excrete more nitrosated amino acids than non-smokers

POSSIBLE ORIGIN OF NITROSOTHIO-PROLINE (S) FOUND IN HUMAN URINE

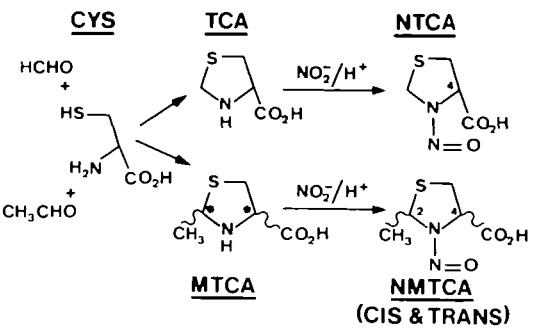

Fig. 1. Scheme depicting the 2-step synthesis of NTCA and NMTCA from L-cysteine, formaldehyde or acetaldehyde and nitrite.

(24 – 26 and Table I), due perhaps in part to higher exposure to salivary thiocyanate (a known catalyst of nitrosation).

Sources of precursors of endogenous nitrosamine formation in man

Further evidence that *in vivo* nitrosation reactions occur in man comes from studies in which piperazine (an anthelmintic drug) and aminopyrine (an analgesic drug) were used as nitrosatable amines (22,23) (Table I). In general, the extent of the nitrosation reaction was shown to be affected by (dietary) intake of nitrate, but smoking and *in vivo* oxidation of ammonia are also contributing factors (48 – 50). Elevated levels of both dimethylamine and NDMA have been found in the intestine of patients with chronic renal failure (51,52) although it is not known whether the presumedly endogenously formed NDMA contributes to the increased cancer risk of those individuals.

Nitrosation of amino precursors present in human gastric juice has been hypothesized to lead to nitroso carcinogens which are responsible for the higher risks of gastric cancer in certain diseased subjects and in populations exposed to high levels of nitrate (53,54). The N-nitrosation of peptides is therefore being studied in order to elucidate conditions under which polypeptides and proteins present in the gastric juice or mucosa might be converted to biologically active N-nitroso derivatives (55,56). Unsaturated lipids capable of serving as nitrosating agents have been isolated from mouse skin after exposure to nitrogen dioxide (57), raising the possibility that similar reactions following exposure to nitrogen oxides from various sources, e.g., tobacco smoke, could lead to formation of N-nitrosamines in other tissues.

The data shown in Table I demonstrate unequivocally that NOC are formed in the human body, even after ingestion of levels of precursors that are considered to be normal. The relevance of such endogenously-formed nitrosamines to human cancer at specific sites, like oesophagus, kidney, stomach, colon and bladder, should now be investigated using combined epidemiological investigations and sensitive methods for exposure monitoring (see below).

Metabolism and formation of DNA adducts

Nitrosamines produce various adverse biological effects, including induction of tumours following metabolic conversion into reactive intermediates which react with various cellular macromolecules; this process is considered as one critical

determinant in the carcinogenicity of nitrosamines. The various forms of microsomal enzymes responsible for this metabolic activation and the nature of the metabolites formed have been described (58–60). Various organs and cells from different species, including human beings, have been shown to be capable of carrying out such metabolic transformation, as determined by the detection of various metabolic products (e.g., carbon dioxide, aldehydes) and/or initial DNA alkylation (e.g., 7-methylguanine).

Table IV shows the results of nucleic acid alkylation obtained following *in vitro* incubation of liver slices from various animal species, including man, with [^{14}C]NDMA. The liver of Syrian golden hamster showed the highest rate of metabolism followed by rat liver; human liver slices have a capacity to activate NDMA that is close to that observed in rat liver; lower activities were observed in the liver of trout and monkeys, these two tissues being relatively resistant to the induction of tumours by NDMA (61,62). These findings *in vitro* parallel those of studies in intact animals, where a similar ratio of the level of DNA alkylation between rat and hamster liver has been observed (63). These results are also consistent with the finding of formation of mutagenic metabolites from NDMA, using a metabolic activation system from livers of different rodent species and from man (64). Liver usually shows a higher capacity than extrahepatic tissues for metabolizing nitrosamines; however, there are some notable exceptions. N-nitrosomethylbenzylamine (NMBzA), a carcinogen specific for rat oesophagus, is metabolized by oesophageal mucosa to a methylating intermediate to a much greater extent in the rat oesophagus than in rat liver, and this effect is independent of the route of administration of the nitrosamine and of preferential uptake into the different organs (65–67). These findings strongly indicate that, in the multistage process of carcinogenesis, tissue-specific metabolic activation is a necessary although not sufficient requirement for tumour induction.

The metabolism of various nitrosamines has also been studied *in vitro* in explants and culture cells from different extrahepatic tissue of human origin (Table V). In the case of NDMA and NDEA, metabolic activation was observed in all the tissues examined, whereas for cyclic and asymmetrical dialkyl nitrosamines considerable diversity was observed. In particular, NMBzA was metabolized to only a small extent or not at all by human oesophagus, in contrast to rat oesophagus and the relative metabolic activity varied among the various tissue explants depending upon the particular nitrosamine. In addition, considerable inter-individual variation, of up to 150-fold, were observed (78) with the human tissue specimens. It should be noted, however, that the number of tissues examined from individual subjects is rather limited and that actual formation of specific DNA adducts has been determined in only a few instances (see Table V). In human liver, formation of 7- and O^6-methylguanine in DNA has been detected (79,80) after exposure to NDMA *in vitro* or *in vivo*, and the pathological changes in the human liver are similar to those observed in rodents following acute or subchronic exposure to this nitrosamine (81,82). Thus, the available data show that tissues from human beings do not differ qualitatively from those from rodent species in their capacity to metabolize nitrosamines.

Following metabolism (or non-enzymic decomposition, in the case of nitrosamides), nitrosamines react with DNA, resulting in the formation of adducts at at least twelve alkylated sites. The relative proportion of alkylation at the N and O atoms of the purine and pyrimidine bases depends upon the alkylating agent (83–86). Starting with pioneering work of Swann and Magee (87), Loveless (88) and Goth and Rajewsky (89), a considerable volume of data now exists which indicates that the formation of O^6-alkylguanine and O-alkylpyrimides is biologically more important than N-7-alkylation of guanine in the initiation of the carcinogenic process by nitrosamines in a specific tissue or cell.

Although it is still under debate whether the persistence of O-alkyl pyrimidines in DNA contributes to the carcinogenic effect as a consequence of their miscoding properties during DNA replication, there is substantial evidence that O^6-methyl- and O^6-ethylguanine are the more critical DNA modification (90,91). The presence of O^6-methylguanine or O^4-methylthymine in synthetic polymers results in the incorporation of non-complementary bases (miscoding) during polyribonucleotide or polydeoxyribonucleotide synthesis *in*

Table IV. Comparative metabolism of NDMA in liver tissue slices from various species, including humans

Species	Relative activity[a]
Hamster (Syrian golden)	100
Rat	65
Man	45
Monkey	6.1
Trout	0.1

[a]Expressed as percentage of the formation of 7-methylguanine and [^{14}C]carbon dioxide observed in hamster (from reference 68).

Table V. Capacity of different human tissue explants to metabolize nitrosamines[a]

Tissue explant	Nitrosamine[b]								
	NDMA	NDEA	NPYR	NPIP	DMPIZ	NMBzA	NEMA	NNN	NNK
Trachea								+	+
Bronchus-lung	+	+	+	±	+			+	+
Oesophagus	+*	+	−			±	−	+	+
Colon	+*	+	+	−	+			−	
Pancreatic duct	+								
Bladder	+		+				+	+	+
Buccal mucosa	+							+	+

[a]Collated from references 69–77. Metabolism [(+) detected; (−) not detected] was determined by measuring the formation of [^{14}C]carbon dioxide, aldehydes or other metabolites and the presence of radioactivity associated with proteins or DNA; formation of specific DNA base adducts was measured in the case of NDMA as indicated by asterisks.
[b]DMPIZ, N-nitrosodimethylpiperazine; NEMA, N-nitrosoethylmethylamine.

Table VI. Modulators of biological effects of nitrosamines in liver and extrahepatic tissues[a,b]

Modulator	Liver				Extrahepatic tissues	
	Metabolism-nucleic acid alkylation *in vivo*	Toxicity	Mutagenicity[c]	Carcinogenicity	Metabolism-nucleic acid alkylation *in vivo*	Carcinogenicity
Protein-restricted diet	↓	↓	↓		↑ Kidney	↑ Kidney
Ethanol	↓ =	↓	↓↑	=	↑ Kidney	↑ Nasal cavity Mice NDEA Oesophagus
	↓ NMBzA			↓ NDEA	↑ NMBzA Oesophagus, Lung	↑ NPYR Nasal cavity Trachea Hamster
Disulfiram	↓ NDMA Mouse NMBzA Rat NDMA, NDEA Rat	↓ NDMA Mouse Rat NDEA Rat	↓	↓ NDMA, DNEA	↑ NMBzA Lung, Oesophagus	↑ NDMA Nasal cavity NDEA Oesophagus NMBzA Oesophagus NDBA Lung
Carbon tetrachloride	↓	↓		↑=	= Kidney	↑ Kidney
Aminoacetonitrile	↓	↓	↓	↓	=	↑ NMBzA Oesophagus
Zinc deficiency						

[a]The results refer to DNMA and rat tissues, unless otherwise specified; however, for clarity, in some cases, the animal species, the tissue and the nitrosamine are specified in full. =; indicates that the modulators have no effect, or contradictory results were observed.
[b]From references 132–138.
[c]Experiments in which post-mitochondrial fractions were prepared from liver of rats treated *in vivo* with the various modulators and used to determine the mutagenicity *in vitro* of NDMA in bacteria, with the exception of one study carried out *in vivo* as a host-mediated assay.

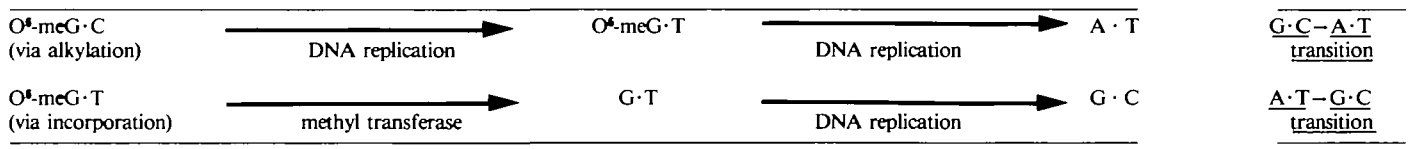

Fig. 2. Mechanisms of mutagenesis induced by O⁶-methylguanine from references 94 and 95.

vitro using a bacterial DNA or DNA polymerase (85). In the case of O⁴-methylthymine, however, its incorporation into DNA of V-79 Chinese hamster cells does not result in the induction of mutation since it behaves like the base cytidine (92,93).

Different mutagenic pathways (Figure 2) have been shown to occur in the case of O⁶-methylguanine: a major one results from direct alkylation of single- or double-stranded DNA, with the occurrence of G·C →A·T transitions, and a minor one results from incorporation of O⁶-methyl-GTP, which yields an A·T →G·C transition *via* the formation of a G·T mispair by the O⁶-methylguanine transferase (94,95). The majority of mutations induced by carcinogens seem to result from replication altered bases ('targeted mutagenesis') and not from 'error-prone repair' (96).

Recently the formation of small amounts of various DNA adducts including O⁶-methylguanine, has been reported following toxic doses of hydrazine (97); this probably occurs through a non-enzymatic DNA methylation by S-adenosylmethionine (98,99). The contribution of this effect to 'spontaneous' mutagenesis and carcinogenesis has still to be assessed; the presence of DNA repair enzymes that very effectively remove damage caused by abnormal methylation appears a prerequisite for cell survival and integrity during evolution.

Recently new *in vivo* data have become available (100–102), which provide some evidence of the relative contribution of O⁴-alkylthymine to the initiation of the carcinogenic process by nitrosamines. It should be acknowledged that the availability of highly specific monoclonal antibodies against these DNA adducts (103) has permitted this type of analysis, which has in turn permitted determination of the non-random distribution of DNA adduct like O⁶-ethylguanine in cell DNA (104).

DNA repair of alkylated bases

Two main processes for the repair of alkylation damage to DNA have been described – one is a DNA glycosylase, which cleave the base-sugar bond of abnormal nucleotide residues, and the other is a O⁶-alkylguanine transferase, which transfers the alkyl group to a cysteine group in a receptor protein leaving an intact base. As detailed reviews have been published recently (86,105), only the more recent developments are discussed briefly below. Remarkable features are the relatively high degree of specificity of these DNA repair processes, and the similarity that has been observed in these major repair pathways between *Escherichia coli* and mammalian cells, including those of human origin,

particularly on the O^6-methylguanine-transferase. The information on repair of O-alkylpyrimidines, although still rather limited, shows that: O^2-methylcytidine and O^2-methylthymine are repaired by the same enzyme (3-methyladenine DNA glycosylase II), which repairs 3-methyladenine and 3-methylguanine; in *E. coli* at least a 2-fold greater release from DNA is observed with the O^2-methylpyrimidines as compared with the N^3-methylpurines (106); O^4-methylthymine is repaired by the methyltransferase that also acts on O^6-methylguanine in DNA in *E. coli* (106,107), athough it has been suggested (108–110), that several methyltransferases may exist. Little is known about the mechanisms of recognition by the enzymes of the various altered bases. In mammalian cells and in *E. coli*, O^6-methylguanine transferase can also function on ethylated, chloroethylated and propylated DNA, although with a lower rate of removal (109,111, and Pegg, unpublished data).

Comparative studies in rodent and in human tissues on the levels of O^6-alkylguanine DNA alkyltransferase show that liver contains a much higher level than do extrahepatic tissues; with the hamster, mouse and guinea-pig liver containing lower amounts than does rat liver (112–116); and adult human liver has much higher levels than rat liver (117–119). Various experiments using different types of human cells in culture also indicate that, in general, human cells have a higher capacity to repair O^6-methylguanine, although human cells deficient in O^6-methylguanine transferase activity in cell culture have been found (120–122). Monkey liver extracts repair O^6-methylguanine and, with a lower efficiency, O^6-ethylguanine, at a rate similar to that detected in human liver (119). Such comparative studies of DNA repair capacity as well as measurements of interindividual variations among human populations appear to be particularly relevant since this approach may allow identification of individuals at high risk of developing cancer as a result of their deficiency to repair these biologically relevant DNA lesions.

It has now been shown clearly that O^6-alkylguanine DNA alkyltransferase is inducible in *E. coli* (105,123) and, more recently, in other systems – *Micrococcus luteus* and *Bacterium subtilis* (124–125), following treatment with low doses of N-methyl-N′-nitro-N-nitrosoguanidine; a similar phenomenon has been observed in mammalian cells *in vivo* (126) and *in vitro* (107,127), although in this case the degree of increased repair capacity is much lower.

Current knowledge about the relevance of these various events (metabolism, DNA damage and repair) to initiation of the carcinogenic process, provides substantial evidence that the induction of tumours in a given organ by NOC is critically determined by a mutagenic event produced by unrepaired DNA lesion (O^6-alkylguanine and, possibly, O-alkylpyrimidines) during DNA replication. These types and sequences of events should be seen and interrelated in the probabilistic context of the multistage process of carcinogenesis.

The relevance of O^6-alkylguanine is also stressed by recent findings (128,129) concerning mammary tumours induced in rats by N-methyl-N-nitrosourea, which indicate that activation of the *H-ras* oncogene is a direct consequence of the interaction of the nitrosamine with DNA. Those authors observed (in tumourous but not in the non-tumourous tissues) a single point mutation – ($G \cdot C \rightarrow A \cdot T$ transition) – which is precisely what one would expect from a defective repair of O^6-methylguanine.

Modulation of nitrosamine carcinogenesis

The tissue specific carcinogenicity of a given nitrosamine is also conditioned by factors other than those discussed above, and it is important to examine the role of the liver in determining the extent to which extrahepatic tissues are exposed to nitrosamines, since the liver plays the major role in the process of activation or detoxification of most of these carcinogens. In fact, after oral administration of very low doses of NDMA, very little or none of this nitrosamine reaches extrahepatic tissues because of the efficient metabolism of the liver (130,131). Thus, interactions of the nitrosamine are strongly determined by the dose of nitrosamine, the rate of absorption from the intestine and by various factors that could modify the metabolic competence of the liver. Experimental studies have shown that a number of factors can drastically change the organotropism of the carcinogenic effect of nitrosamine (see Table VI).

Administration of a protein-restricted diet to rats results in a decrease in liver toxicity and DNA alkylation and, as a consequence, an increase in DNA alkylation in the kidney. This change in the pharmacokinetics of NDMA is associated with a dose-related increase incidence of kidney tumours in rats fed a protein restricted diet (139).

Ethanol has also been shown to decrease the metabolism and toxicity of NDMA and some other nitrosamines in the liver and, in parallel, to increase tumour incidence in the oesophagus, kidney and nasal cavities. It should be stressed that this effect is observed with very low concentrations of ethanol (< 1 mmol/l) and that some observations in man are consistent with the experimental data (see 134 for more details). Table VI shows that other agents modify the various biological effects of nitrosamines in the liver and in extrahepatic tissues. These observations have considerable bearing in the planning and analysis of epidemiological studies aimed at determining whether there is a causal association between exposure to nitrosamines and some types of human cancer.

Recent methodological developments using immunoassays with specific antibodies (103) have made possible the detection of low levels of DNA modification (e.g., O^6-alkylguanine). Application of this approach in human studies resulted in the detection of these (this) DNA modification(s) in oesophageal tissues from individuals of a population at high risk of oesophageal cancer and for whom there is some evidence of exposure to nitrosamines (170).

Epidemiological studies and combined laboratory/epidemiology investigations to link NOC and their precursors with human cancers

NOC in tobacco carcinogenesis

NOC occur in fermented tobacco products and tobacco smoke, and this is the greatest and most widespread source of human exposure presently known, except for some occupational exposures (see Table II). Several volatile and non-volatile nitrosamines have been detected, including four tobacco-specific nitrosamines (TSNA) that occur at high concentrations in tobacco smoke and at even higher concentrations in snuff and chewing tobacco (30,140,141). The concentration of TSNA in particular was shown to be related to the nitrate content of the tobacco product. Levels of volatile nitrosamines are considerably higher in side-stream smoke than in main-stream smoke; this raises the question of

whether these volatile nitrosamines contribute to the carcinogenic risk associated with passive exposure to smoke (142).

The well established correlation between exposure to tobacco smoke and risk of cancer of the upper respiratory tract (143) strongly suggests that TSNA contribute to the induction of these malignancies. Supportive evidence comes from carcinogenicity and metabolism studies in experimental systems (144): the principal organs of experimental animals affected by TSNA are lung, trachea, oesophagus and nasal cavity; these organs include the major target sites at risk in humans who smoke.

Of the TSNA, NNK has been shown to be the strongest animal carcinogen (144); it is noteworthy that NNK forms a metabolite that can be reconverted to the parent compound *in vivo*, thus leading to prolonged exposure. In metabolic studies in experimental animals and also in human tissues *in vitro*, NNK and NNN were shown to be readily converted into electrophiles that can react with DNA; metabolic studies of buccal mucosa, oesophagus, bronchus, lung and liver of human origin indicate that the metabolism of NNN and NNK follows routes of activation very similar to those in rodent tissues (144).

Recently, an association has been confirmed between human cancer and snuff dipping in some southern states of the USA (145). Tumours usually arise at the site in the mouth where such tobacco wads are retained. Since no chemical carcinogen other than TSNA [at concentrations 100 times higher than in other products (140,141)] has been detected in snuff a direct association between exposure to N-nitrosamines and induction of cancer in humans could be assumed in this specific situation.

There is an established correlation between oral cancer and chewing of betel quid (which often contains tobacco) in India and other South-East Asian countries (145,146). It has been shown that nitrosation *in vitro* of arecoline, a betel nut alkaloid, leads to the formation of three N-nitroso compounds, of which N-nitroso-N-methyl-proprionitritile is strongly carcinogenic in experimental animals (147). Thus, carcinogens arising from the nitrosation of betel nut- and tobacco constituents should be investigated further to establish their role in oral cancer produced in betel quid chewers.

Other studies on human health effects related to presumed exposure to NOC and precursors

Further epidemiological studies are being carried out to examine possible relationship between adverse health effects in humans and presumed exposure to NOC and their precursors; however, only in rare cases have NOC been identified as the disease-related agents, nor has exposure of the study populations/subjects been quantified. Some of the results presented below, however, are suggestive of such a relationship and require further investigation.

Epidemiological evidence has been presented linking presumed exposure to NOC and precursors to the development of cerebral tumours in humans (148). Consumption by pregnant women of large amounts of Icelandic smoked mutton has been associated with the induction of diabetes in their male progeny. This adverse effect was also produced in male offspring of mice fed Icelandic smoked mutton after mating (149). It is not clear whether the high (p.p.m.) levels of N-nitrosothiazolidine and NTCA that have been detected in smoked meat products (including Icelandic mutton) are related to the induced diabetes. In a case-control study of diet and stomach cancer recently conducted in Canada, it was found that the average daily consumption of nitrite (as well as of carbohydrates) was associated with increasing trends in risk, while dietary fibre decreased the risk of gastric cancer (A.B.Miller, personal communication).

Endogenously produced nitrosamines are hypothesized to be associated with an increased risk for bladder cancer in bilharzia-infested populations in certain North African countries (150). Secondary bacterial infections of the bladder are regularly associated with bilharziasis; under such conditions, nitrosamines can be produced because of increased nitrate to nitrite conversion and the acidic pH of the urine. Such *in situ* formed NOC could initiate carcinogenesis in the bladder urothelium.

Occupational exposure to NOC

In certain industries (Table II), such as leather tanning, metal working and rubber and tyre manufacture, relatively high concentrations of volatile NOC have been found frequently in the ambient air and in grinding fluids (151–153). NDElA, a carcinogenic nitrosamine which has been found in cutting fluids used in the metal working industry, can be monitored in the urine of exposed workers (153), and this NOC has also been detected in some cosmetic products (Table III). Because such exposures can be (and have been) reduced, continued epidemiological surveys, especially in the rubber industry, should indicate whether the preventive measures adopted were effective; but, clearly, more epidemiological investigations are warranted to assess the risk from occupational exposure to certain N-nitrosamines.

Nitrosamines and cancer of the upper digestive tract and bladder

Gastric cancer and achlorhydria. Patients with chronic atrophic gastritis (CAG) or pernicious anaemia or those who have undergone (Billroth-II) gastrectomy are at increased risk for stomach cancer (53); it has been postulated that the achlorhydric stomachs of such patients may provide a suitable milieu for the intragastric formation of carcinogenic NOC because of the presence of large numbers of the bacteria that are involved in the conversion of nitrate to nitrite and subsequent nitrosation of amino precursors *in vivo* (53,54). In fact, there is now evidence that fasting gastric juice of such patients frequently contains higher levels of nitrite and of total NOC [as determined by the method of Walters *et al.* (15)] than that of healthy subjects (154,155). After four weeks of treatment with ascorbic acid, a significant reduction was found in total NOC in the gastric juice of hypochlorhydric subjects (156). A pilot study was carried out using the NPRO test to compare excretion of nitrosated amino acids in subjects with histologically ascertained CAG (Table I) *versus* that in healthy controls (26). Urinary NPRO levels were dependent on gastric pH (maximal yields at about pH 2), but CAG patients (at a presumably higher risk for gastric cancer) did not appear to excrete more NPRO. However, the contribution of a bacterial enzyme-catalysed nitrosation reaction needs further exploration; it has been claimed that an *E. coli* strain is capable of catalysing nitrosamine formation at neutrality from nitrite and an amine *in vitro* (157). These data clearly indicate that endogenous nitrosation does occur in the human stomach, but its relation to the induction of upper gastro-intestinal cancer remains to be proven.

On the basis of anecdotal clinical findings in a number of cases of carcinoma of the stomach in gastric ulcer patients who had received cimetidine for 6–24 months, concern has

been raised about the effect of H_2-receptor blockers, like cimetidine or ranetidine (158,159). Millions of humans take these drugs, which produce hypochlorhydria (gastric pH above 4) and gastric bacterial overgrowth, resulting in a higher conversion of nitrate to nitrite. The latter, in combination with dietary or pharmacological amines could produce higher concentrations of NOC than in non-treated patients or in normal, healthy persons. Although it has been reported that subjects treated with cimetidine frequently have an elevated level of total NOC in their gastric juice (155,158) and that ranetidine treatment increases the urinary excretion of NTCA (159), the relevance of these findings is under debate (160). The hypothesis that most of a dose of cimetidine is N-nitrosated *in vivo*, yielding a nitroso compound with carcinogenic properties, has not received support from experimental studies (161 – 163).

Oesophageal cancer in Northern China. Extensive research, which began in 1972, in the People's Republic of China has suggested that N-nitroso compounds and their precursors may be among the etiological factors involved in the causation of oesophageal cancer in certain provinces in Northern China (164). Preformed NOC, nitrite and nitrate were detected in a number of food items and in drinking-water. In a recent pilot study, the excretion of urinary nitrosamino acids by inhabitants living in high-risk (Lin-Xian) and low-risk (Fan-Xian) areas for oesophageal cancer was compared (27). Lin-Xian subjects excreted significantly more nitrate and N-nitrosamino acids than those living in Fan-Xian (Table I). When Lin-Xian subjects were given ascorbic acid (100 mg after each meal), the level of urinary N-nitrosamino acids was reduced to the levels found in Fan-Xian. Ascorbic acid, an efficient inhibitor of endogenous nitrosation, should now be examined in intervention trials.

Conclusions and public health implications

Although a causal association between nitrosamine exposure and human cancer has not yet been rigorously established, the recognized association between exposure to nitrosamines in unburned tobacco products and oral cancer in humans (145) is as close as one is likely to get in epidemiological studies of this class of carcinogen. In addition, biochemical, pathological and experimental data provide little evidence that humans are resistant to the carcinogenic action of NOC, from either preformed or endogenous sources: a large number of animal species are known to be susceptible to cancer induction by NOC, and no species has been found to be resistant. Other supportive evidence includes the capacity of human tissue preparations, cells and short-term organ cultures, including liver, buccal mucosa, oesophagus, bronchus, liver, colon and urinary bladder for metabolic activation of a number of nitrosamines into electrophilic and DNA-binding species (Table V). These results indicate that metabolic pathways are often qualitatively and quantitatively similar in animals and humans. Although quantitative differences exist between rodents and humans in repair of DNA alkylation damage, the mechanisms of repair of this damage appear to be the same. Recently, malignant transformation of human pancreatic epithelial cells by NDMA has been reported (165).

Earlier reports on the acute toxicity of NDMA in occupationally exposed workers (liver necrosis and cyrrhosis) were confirmed in recent criminal poisoning cases with the same compound, whereby the victims died of liver necrosis and cyrrhosis (81 – 82,166); the same symptoms are seen in rodents after exposure to high doses of such nitrosamines. Circumstantial evidence that human cancer can be induced by NOC was also described by various authors (167 – 169), who observed acute non-lymphocytic leukaemia in patients treated for brain tumours with cytostatic nitrosourea-derivatives.

There is not only an association between snuff-dipping, betel quid chewing and buccal cancer, but there is also an established correlation between tobacco smoking and upper respiratory-tract cancer in humans; there are good grounds to believe that because of their organotropic carcinogenicity for the respiratory tract, TSNA are also involved.

The human stomach is another site in which nitroso compounds have been implicated in the causation of cancer. There is epidemiological evidence from several countries relating elevated intake and levels of environmental nitrates with stomach cancer; and it is now proven that NOC are formed from ingested precursors in the human stomach (Table I).

The evidence accumulated to date, indicates that the levels of nitrosamines found in man's environment may be involved in the causation of human cancers. However, it is perhaps difficult, or impossible, to demonstrate in the general population a cause/effect relationship between exposure to low levels of nitrosamines and the incidence of certain cancers, due to the insensitivity of the epidemiological instruments available today and to the lack of truly unexposed populations that could be used as controls.

In addition, individual exposure to endogenous nitrosamines is affected by dietary components, and host susceptibility to nitroso carcinogens may be modified by host factors and a variety of modifying chemicals, man-made or of natural origin. Micronutrient deficiencies may have marked effects on NOC-induced carcinogenicity, both qualitatively (target organ) and quantitatively (incidence). Therefore, low levels of exposure to nitrosamines may be sufficient to cause cancer in cases where dietary deficiencies (vitamins, trace elements, anti-oxidants) or excesses (high-salt diet) predominate in determining whether an individual human subject develops cancer or not.

For all these reasons, we should be prepared to work out means for preventing the induction of cancer in humans by NOC and implement such measures. There are various ways in which exposure of humans to NOC can be decreased: (i) modification of habits (for example to stop using tobacco products); (ii) changes in technological processes (like meat-curing, rubber manufacture, drug synthesis); (iii) hygienic and regulatory measures in occupational settings; (iv) decreasing the levels of precursors in the environment (nitrate, nitrite, nitrogen oxides, easily nitrosatable amines that give rise to carcinogenic NOC); (v) possible use of numerous nitrosation inhibitors (such as ascorbic acid, α-tocopherol and certain polyphenolic compounds); and (vi) remedying micro-nutrient deficiencies, which are now being recognized as potentiating risk factors, in high-risk areas where NOC are thought to be among the etiological agents responsible.

Thus, lowering the body burden of nitroso carcinogens by systematic application of ultra-sensitive detection and monitoring methods for nitrosamine exposure and eliminating potentiating risk factors – as verified by preventive intervention trials – should reduce a portion of occupational, diet-related and environmental life-style cancers.

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