Potential genotoxic hazards associated with medicines and other manufactured products

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Abstract

Medicinal products sometimes have adverse effects that concern a small but significant proportion of patients. Since a person can fall ill at any time, special statistical analyses of large numbers of adverse event reports are needed to establish whether a particular type of symptom could have been caused by a particular treatment. Worldwide pharmacovigilance programs exist for this purpose. Detecting quantitatively minor genotoxic causes of common types of cancer would be particularly difficult, because the background incidence is high and variable, and symptoms appear a long time after exposure. Anticipatory testing may not be sufficiently sensitive to detect genotoxicity risks that are considered significant (officially, one case per 100 000 patients on the basis of life-long drug treatment). While carcinogenic risks due to ionising radiation have now been established for exposures down to those encountered with diagnostic x-rays, it is

rarely possible to make similar calculations for exposure to chemical carcinogens, even when the existence of a hazard is known.

Many natural and artificial carcinogens have been identified, and they are considered more or less seriously, depending on public and personal attitudes to them. The subject of this document is the possibility that further significant exposures to genotoxic (and therefore likely carcinogenic) compounds of synthetic origin may have been either unsuspected or ignored.

Three possible examples of potential pharmaceutical genotoxic hazards are presented, and the third of these may have ramifications for non-pharmaceutical applications of the products concerned. In each of these cases, it could be envisaged that chemical compounds may react with DNA in a manner that is known to have genotoxic consequences.

1. Unexpected chemical reactivity in a series of drug substances having the *ortho*-alkoxybenzamide structure is alerting for genotoxicity.
2. Various nitro-heterocyclic antibiotics have known alerts for genotoxicity; they are banned for administration to food animals but not to humans. However, in view of the proliferation of microorganisms resistant to other antibiotics, these products could possibly provide leads for new products for use as a last resort.
3. The semi-synthetic carbohydrate ethers, widely used as drug excipients and for many other purposes, may present a genotoxic hazard that does not seem to have been discussed.

It is likely that cases such as these could be brought to light only by some form of chemical serendipity. Unfortunately, there does not seem to exist any official body to which scientists may report such concerns in a responsible manner. A probable reason for lack of attention to this subject is its interdisciplinary nature; no-one feels confident enough to discuss and evaluate every aspect. No-one is able to estimate the risk associated with a potential hazard that has not yet been investigated, nor to decide whether a question merits investigation.

Some background information is given in an appendix:

– The chemistry of relevant types of chemical reactions of xenobiotics with DNA is presented, partly to demonstrate serious apparent deficiencies in some published research and review papers. Numerous studies have attempted to correlate electrophilic reaction kinetics and mechanism with DNA adduct formation and consequent mutagenesis. The reagent 4-(4-nitrobenzyl)pyridine (NBP) is commonly used as a surrogate for DNA, but much

reported experimental data is questionable because of chemical artefacts that have been described but are generally neglected.

– Some electrophilic mutagens that react with DNA have linear dose-response relations. Others, including many to which humans can be exposed, have different reaction mechanisms; they show non-linear responses indicating relatively small effects at low doses. Consequently, they are considered to present risks that are lower than would be estimated for a linear relation. The difference has been ascribed to (demonstrated) saturable DNA repair activity, which is more or less effective depending on the sites of DNA-adduct formation; site-specificity is a function of the reaction mechanism. Surprisingly, despite the importance of the subject, the literature on DNA repair does not seem to have been evaluated in the context of known toxicokinetic factors. In particular, depending on the reaction mechanism, an electrophile may or may not be scavenged in a saturable manner by endogenous nucleophiles.

1 Introduction

1.1 Genotoxicity and cancer

The incidence of cancer in humans, corrected for demographic factors, has been increasing during the last few decades. This could be interpreted as Nature's belated feedback response to overpopulation, but most people would rather die of something else.

During the period concerned, exposure to synthetic and semisynthetic chemicals has also been increasing. It's facile and fashionable to conclude that "chemicals" are a major cause of changing patterns of non-infectious disease. It's also easy to forget that some natural metabolites such as the aflatoxins and the aristolchic acids are potent carcinogens that may occur in food and herbal "remedies"; competition between organisms often amounts to chemical warfare. Nevertheless, numerous synthetic compounds, including some drugs, have chemical structures that are alerting for genotoxicity, of which cancer is the endpoint usually evaluated. This document is primarily concerned with potential genotoxic hazards associated with medicines, but the arguments extend to some other applications of synthetic chemicals.

Structural alerts do not cover all potential mechanisms of genotoxicity, but they are relatively amenable to investigation in a field of study that presents remarkable difficulties. A compound (or a metabolite of it) with a structural alert may react directly with DNA or its associated proteins,

leading to a transmissible genetic defect. To limit the number of explanatory digressions, we assume that such genotoxic compounds are electrophiles that react with known negatively-polarised sites on DNA (Appendix A.1). This kind of reactivity can usually be predicted and evaluated for a known compound or substance under study from knowledge of the chemistry, by screening for structural alerts (perhaps aided by software), and by direct testing with various model nucleophilic reagents (including DNA or isolated fragments of DNA). These methods are backed up by a standardised battery of *in vitro* and *in vivo* biological tests, which also cover other genotoxic mechanisms. Testing in biological systems is, however, expensive and not very sensitive. It is not always possible to declare that a compound is not hazardous at the required level of risk, bearing in mind the limited sensitivity of experimental methods and the required low level of lifetime risk.

1.2 What cancer risks are considered acceptable?

The limit of acceptable risk is decided according to the situation. The World Health Organisation's recommendation for genotoxic carcinogens in drinking water illustrates the difficulties (reference [1, Chapter 8]):

> Guideline values are conservatively presented as the concentrations in drinking-water associated with an estimated upper-bound excess lifetime cancer risk of 10^5 (or one additional case of cancer per 100 000 of the population ingesting drinking-water containing the substance at the guideline value for 70 years). This value does not equate to the number of cases of cancer that will be caused by exposure to the substance at this level. It is the maximum potential risk, taking into account large uncertainties. It is highly probable that the actual level of risk is less than this, even approaching zero, but risks at low levels of exposure cannot be experimentally verified.

As stated by the WHO, these limits may seem cautious, particularly when compared to the current lifetime incidence of cancer, which is said to be more than 1 in 3. However, humans are exposed to numerous natural and synthetic carcinogens. An opinion not held by all experts is that the total risk attributable to chemical causes should be related to the sum of the individual exposures, weighted for potency.

A lower risk such as one per 10^6 may be required in other situations, for example where exposure is avoidable or there is no significant benefit. Some of the carcinogens in drinking water are due to sterilisation processes and are considered acceptable up to a certain level in view of the far greater

risks associated with microbial contamination. Since pharmaceuticals are presumed to be beneficial, the allowed lifetime risk for genotoxic impurities in drug substances has been set to the same as that for drinking water (one per 10^5); although such impurities are avoidable in theory, there are economic and scientific constraints.

1.3 Relating exposure to a genotoxic chemical to the risk of cancer

A few years ago, regulatory authorities began to require the systematic study of potential genotoxic impurities in drug substances, and much has been written about the subject. One of the subjects of this document is the relatively neglected question of what to do about the intrinsic genotoxicity of certain drug substances other than anti-cancer products. Although some examples are well known (such as the nitrofuran antibiotics), others have not been studied at all, and the subject does not seem to have been systematically reviewed.

In practice, estimating chemical genotoxic potencies is exceedingly difficult, much more so than with ionising radiation. As a result, a TD50 value - the amount that causes 50 % excess tumours after prolonged exposure - is known only for a relatively small number of toxic chemicals that are commonly used or encountered (for example in drinking water). Consequently, regulatory authorities have adopted a "default" exposure limit for chemicals that are patently genotoxic, but which can not economically be studied exhaustively. An example for which regulations or guidelines have been issued recently recently is the case of drug impurities that are found to be genotoxic. The manufacture of many if not most synthetic chemical products such as drug substances involves highly toxic chemicals, and therefore a large number of such potential impurities must be screened for. As mentioned, the present document is concerned largely with potential genotoxic hazards that are intrinsic to drug substances. There appears to be a regulatory vacuum here, and products seem to be prescribed with less discernment than is the case with diagnostic ionising radiation.

Because of all the uncertainties, the daily exposure to a genotoxic chemical considered to entail the conservatively-estimated lifetime risk of 10^{-5} described above is low: 1.5 μg per compound. I understand that this limit is based on toxicities typical of common alkylating agents that are used in synthetic chemistry, as experimental mutagens and for cancer chemotherapy (the arguments are quite complex, especially for chemically reactive compounds). Because of the high and fluctuating normal incidence of cancer in

mice and men, it is not possible to determine directly the dose of a chemical that causes one attributable cancer per 10^{-5} lifetimes. We know, however, that a single (or divided) dose of 400 micrograms/kilogram (a few tens of milligrams) of mustine, the earliest cytostatic alkylating agent, causes a notable incidence of new cancers. The exact figure is unknown, but it may be a few percent within the decade that follows treatment. Extrapolating the data to low doses is a controversial matter, but it can be argued that, at least as a precaution, unwanted exposures to such potent substances should be limited to no more than a few micrograms per day.

Some carcinogens are extremely potent, but these are considered sufficiently rare among synthetic chemicals to be excluded from the present discussion. Possibly of more serious concern are compounds that are too weakly potent for significant toxicity to be revealed by current test methods, because of weak chemical reactivity, the presence of uncharacterised impurities, or chemical interactions that generate traces of potent carcinogens.

A serious limitation of the "default" limit of 1.5 μg per day, not sufficiently taken into account by the pharmaceutical regulatory authorities, is that the potencies of the small number of carcinogens that it has been possible to study in sufficient detail range over five or more orders of magnitude. Manufacturers protest at the expense of meeting stringent specifications for drug impurities that would likely turn out to be weakly toxic if it were possible to obtain quantitative genotoxicological data. Jackson *et al.* [2] have proposed a potency ranking for a large number of carcinogens, taking a pragmatic approach to numerous variable factors such as species, affected organs and mode of administration. Whatever the validity of this proposition, it does provide a graphic illustration for the lay reader of the range of potencies. The proposed ranking [2] also provides some structure-activity information for well-studied classes of carcinogens such as the halocarbons. Unfortunately, although notions of risk analysis are beginning to be introduced in the field of the safety of medicines, there exists as yet no general ranking or classification scheme based on scientific rationale that could be used to justify exposure limits for genotoxic impurities that are much higher than the default value.

I became interested in the subject of genotoxicity in about 1990 when, as an analyst working for a pharmaceutical company, I was asked to develop a method for the determination of a reactive electrophilic impurity in a drug substance. Some of the analytical methodology developed by our team was published. One of these papers describes a false regulatory alert that could, perhaps, have been avoided [3].

1.4 Regulatory and other influences

Until recently, chemical safety regulations were usually introduced in reaction to incidents and epidemiological studies in which there was strong evidence for a cause-effect relationship. The European legislation covering substances that are carcinogenic, mutagenic or toxic for the reproductive system (CMR) is one example of this approach. Many of the listed CMR substances are genotoxic (they alter the genetic code of daughter cells when cells divide), though there are other modes of action.

More recently, legislation has become anticipatory, as exemplified by the European REACH procedures for industrial chemicals, and the international regulatory initiatives concerning genotoxic impurities in medicines. Strangely, the pharmaceutical regulations cover (at least for the moment) only impurities in drug substances, and there is little discussion of excipients or of the interactions that can occur in complete formulated products. Many drug excipients are high-quality versions of industrial products that have numerous other uses; the economic importance of these products could lead could lead someone of an enquiring mind to ask inconvenient questions about the underlying reasons for their exclusion.

A legislative approach to a subject as complex as genotoxicity may prove misleading when one is searching for subtle potential hazards that may have escaped attention. Benzene receives particular attention in the CMR legislation because it was once used as a degreasing agent, particularly effective for hand washing. This aspect of the legislation has become obsolete because benzene is now difficult to obtain, even as a reference substance for calibrating analytical methods. On the other hand, various alarmingly genotoxic chemicals escape regulatory attention because their only industrial application is as mutagens, for example for the purposes of plant breeding.

European authorities do not appear to have procedures whereby informed individuals can submit concerns about the safety of drugs or other substances, apart from the reporting of manifest drug side-effects. Possible consequences of regulatory unresponsiveness are illustrated by a recent case in which a rare but severe specific cardiac side-effect of Mediator (benfluorex) was acted upon only through the perspicacity and persistence of one doctor. Carcinogens that are weakly potent should be particularly worrying, because one can never be certain to have detected all possible hazards, and it is quite impossible to link an individual case to an exposure that may have occurred years previously and that has a low but significant probability of causing cancer.

Reluctance to investigate potential hazards appears to be just one of the more subtle aspects of a network of industrial influence. Since retiring, I contributed to a correspondence about possible influences on Royal Society

of Chemistry (UK) food and agriculture policy [4]. I also had to approach the publisher of a sponsored scientific journal to claim the right to reply to some articles from industrial sources that discussed our work [5]. On the other hand, it is quite legitimate for firms competing in a given field to collaborate on technological and regulatory matters, and also to protect their interests when unscrupulous competitors might take liberties with workplace safety; the manufacture of carbohydrate ethers (some of which are used as excipients, see below) is a hazardous activity. Also, we must accept that if every possible hypothetical objection concerning safety were allowed to retard development, very few products incorporating chemicals (including pharmaceuticals) would reach the market. The counter-argument is that industrial pressure and infiltration of sources of information, combined with regulatory laxness or worse, have led to the widespread use of CFCs, promotion of tobacco, lead in petrol, lead and reprotoxic solvents in paints, and so on.

In this document I will present three cases I have encountered, which do not seem to have been investigated as thoroughly as they could have been. In each of these, there seems to be some probably small risk of human genotoxicity, but it is impossible for an individual working alone to evaluate this risk.

1.4.1 Note on the literature on electrophilic reactivity and genotoxic endpoints

The literature on the relations between structure, electrophilic reactivity and genotoxicity is vast, and the articles I have been able to obtain are quite difficult to follow. One reason for the difficulty is that a lack of interdisciplinary comprehension has led to some fundamental misunderstandings; some of these are discussed in the Appendix. These difficulties could lead someone new to the subject to doubt the validity of much that has been written. Dubiety may be unnecessarily compounded by the fact that we are discussing for the most part hazards that are associated with industrial chemistry and pharmacy. It is, therefore, natural for research in the field to be motivated, and to some extent oriented, by industrial concerns. For example, some recent studies of dose-response relationships were quite legitimately sponsored by − declared − vested interests [6], [7].

2 The *ortho*-alkoxybenzamides

Numerous drug substances are benzamides with a side-chain having a basic aliphatic function. A possibly surprising number of these also have an *ortho*-

alkoxy group (nearly always methoxy). Nineteen *o*-alkoxybenzamides that reached a stage of development where an International Nonproprietary Name (INN) was assigned are shown in Figures 1 and 2.

The compounds are mainly antiemetics (metoclopramide) or atypical neuroleptics that act as dopamine receptor antagonists. Among the latter, sulpiride, sultopride and amisulpiride are also agonists of the recently-identified GHB receptor. One of the neuroleptics, veralipride, was at one time sold for the treatment of menopausal symptoms, despite the known irreversible side-effects of this class of compounds. The practice in some countries of using the trade names of formulated products (Agreal and Agradil in this case) instead of the INN (veralipride) may, as with benfluorex, have misled prescribers as to the therapeutic class of the drug substance.

Some years ago, during research on the degradation of some *o*-methoxybenzamides, I noted a disproportionation reaction in which the methoxy function methylates the tertiary side-chain nitrogen of another molecule (Figure 3).

Such alkylating reactivity is alerting for potential genotoxicity. Degradation was not rapid enough to compromise the shelf life of the medicinal products according to standard criteria, and it may have been reasonable at the time to consider the reaction toxicologically insignificant. On the other hand, reactions that occur during storage are not necessarily good models for the situation *in vivo*. Moreover, since members of the series have various electron-withdrawing groups *para* to the benzamide and alkoxy functions (R_2, R_3), they would not all be expected to have similar reactivities.

Aromatic ethers are not usually considered to be alkylating agents except under severe conditions used in industrial processes or for analytical purposes. However, they are activated by an adjacent benzamido group, presumably via hydrogen bonding, and the reaction is of synthetic utility [8].

According to this report [8], the corresponding *o*-alkoxybenzoic acids are considerably more reactive than the benzamides. The acids are synthetic intermediates of the amide drug substances, and are usually specified impurities. Consequently, they ought to be considered as genotoxic impurities. The sticking point for an open and rational discussion is that while, for several reasons, *o*-alkoxybenzamides and even *o*-alkoxybenzoic acids seem unlikely to be highly potent genotoxins, it would be difficult – exactly because of this – to obtain real experimental data that would justify allowing a daily dose greater than the default limit of 1.5 μg.

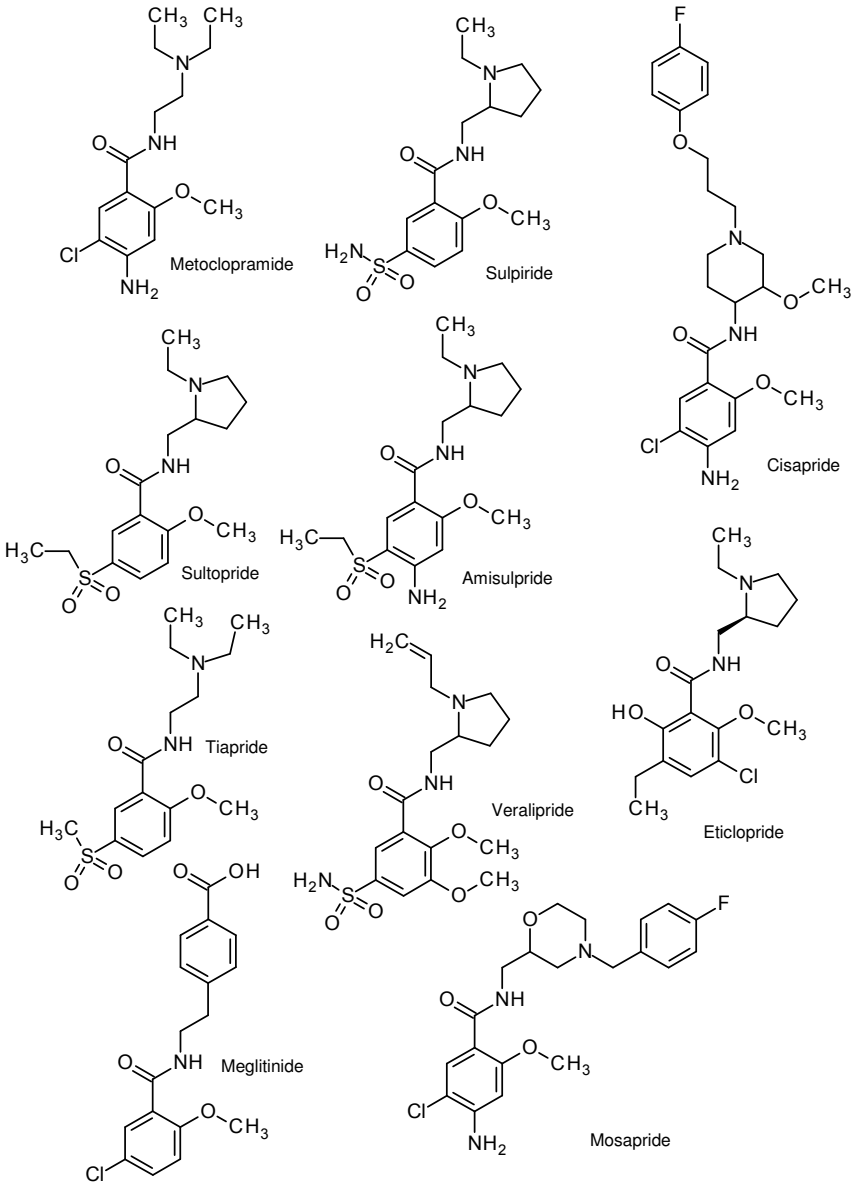

Figure 1: *Ortho*-alkoxybenzamides

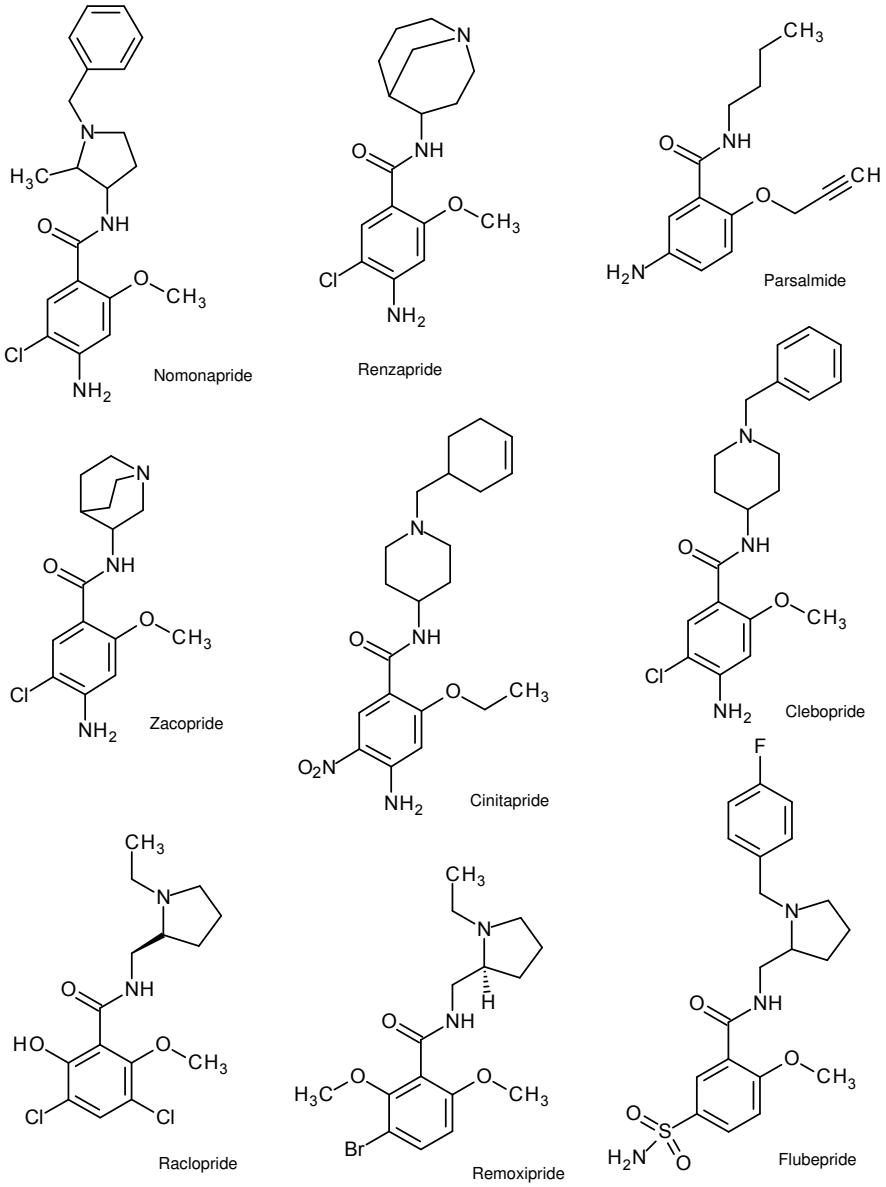

Figure 2: *Ortho*-alkoxybenzamides (continued)

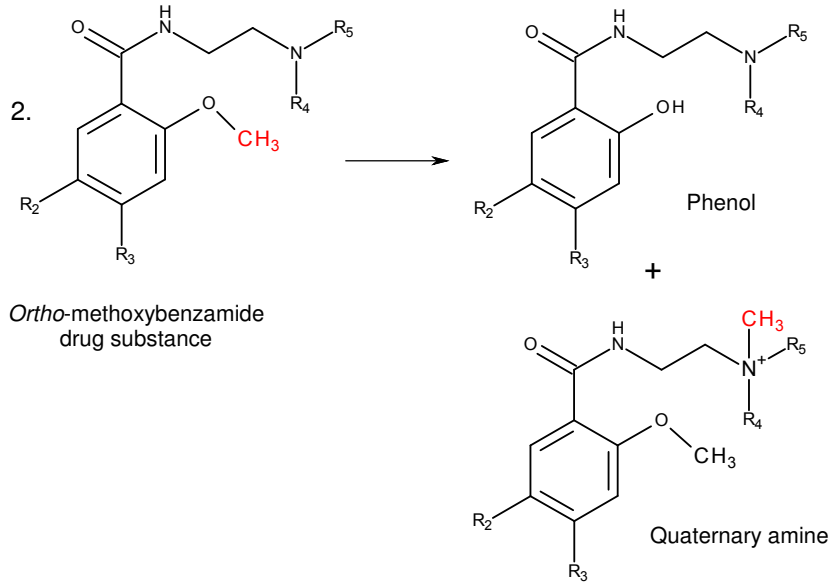

Figure 3: Disproportionation reaction of *ortho*-methoxybenzamide drug substances. One molecule methylates the aliphatic amino side-chain of a second molecule. This degradation occurs during stability studies carried out under standardised storage conditions. R_1 (not indicated): O-methyl, shown in red; R_2, R_3: various functional groups; R_4, R_5: alkyl, may be cyclic.

2.1 Reaction mechanism; natural N-methylation of DNA, and DNA repair

The alkylation reaction mechanism of these compounds is likely to be close to "pure" SN2 (Appendix A.1.2). This means that if DNA is indeed alkylated by the *o*-alkoxybenzamides, the reaction will be regiospecific; only certain ring nitrogens of the DNA bases will be modified, assuming that the compounds penetrate as far as the nucleus. For most of the benzamide drugs, the pattern of alkylated DNA is, therefore, likely to be similar to that produced by spontaneous reaction with S-adenosylmethionine (SAM). SAM is the cofactor in numerous enzymatic methylation reactions, including the epigenetic methylation at C-5 of certain DNA cytosines 10. Since SAM is a thionium ion, it also has weak spontaneous reactivity at SN2 nucleophilic sites. All life forms have enzyme systems that detect and remove "unwanted" N-methyl groups from "SN2" sites of DNA bases. The base-excision-replacement repair mechanism (BER; replacement of a defective segment of a DNA strand) also operates. These repair mechanisms entail considerable metabolic resources; a mystery of molecular biology is why Nature has not evolved a less spontaneously reactive methylating cofactor than SAM. Like some computer operating system, it looks as though our DNA-based life system may have been launched before it was quite ready.

Evaluating any possible genotoxicity of *o*-alkoxybenzamides and the corresponding carboxylic acid impurities is, therefore, a particularly complicated matter. Toxicokinetic differences *in vivo* are likely to render difficult any comparison with the relatively much more reactive alkylating agents that are usually used as reference compounds. It is certain, however, that (unlike the reference genotoxins) weakly reactive electrophiles that reach the cell nucleus will not be significantly degraded, for example by reaction with water and other endogenous nucleophiles.

The anti-emetic metoclopramide (Figure 1) is known to be genotoxic in cultured mammalian cells [9]. The authors of this report would not, however, have been aware of the alkylating reactivity. They could not establish whether or not reactive metabolites are the cause; like some other compounds of this series, metoclopramide is a primary aromatic amine, which forms the well known electrophilically reactive oxidative metabolites. The authors proposed that the observed genotoxicity may not be clinically relevant at the doses used for the treatment of ordinary gastrointestinal disorders. A possible gap in this argument is that tissues may become relatively vulnerable to genotoxins under pathological circumstances such as inflammation[1]. The

[1]This argument will be expanded in the section on nitrofurans and other nitro-

risk/benefit ratio was considered to be favourable in the treatment of vomiting caused by chemotherapy. This analysis is relevant also to the neuroleptic benzamides, as the mortality among patients with major psychoses is high. I add that clinical considerations could also justify the number of neuroleptic benzamides on the market, bearing in mind particularly that the last word has not yet been said on their mode of action.

Finally, the almost universal presence of the *o*-methoxy group in the benzamides that were developed seems surprising in view of the range of different substituents that medicinal chemists introduce when optimising a lead. I propose on the basis of no experimental evidence that the action of these drugs could possibly involve irreversible inactivation of the target receptor by an alkylation reaction, which could be favoured by the particular stereochemical configuration associated with high affinity receptor binding. The O-methyl group would be considerably more reactive than other simple O-alkyl functions. This idea arose because I once played a minor role in studies of the alkylating agent R(-)-N-(2-chloroethyl)norapomorphine as an irreversible antagonist of dopamine receptors [10].

3 Nitrofurans and other nitro-heterocyclic drugs

The development of microbial antibiotics such as penicillin and derivatives was concurrent with that of various synthetic small-molecule antibiotics (for example the sulfonamides (1935) and trimethoprim (1950s)). Starting in the 1940s, nitrofurans (Figure 4) and some other nitro-heterocycles (Figure 5) were introduced. An extensive review of the nitrofurans (including the regulatory situation) was published in 2008 [11].

Each of these compounds has at least one obvious structural alert for genotoxicity. Aromatic nitro-compounds are metabolically reduced, giving essentially the same range of reactive products as are formed by oxidative metabolism of the corresponding aromatic amines. Most compounds of this class have been shown to be genotoxic and/or carcinogenic to some extent, but as is usual in this field not all reports satisfy all regulatory requirements. Nitrofurantoin, but not nifuroxazide, was reported to be weakly mutagenic in vivo in the "Big Blue" transgenic mouse assay [12].

The nitro-heterocyclics are broad spectrum antibiotics, still important because they are also effective against some protozoans and other parasites, and because they do not readily induce drug resistance. One niche application is for coating sterile catheters. Nifuroxazide is an over-the-counter

heterocyclic drugs

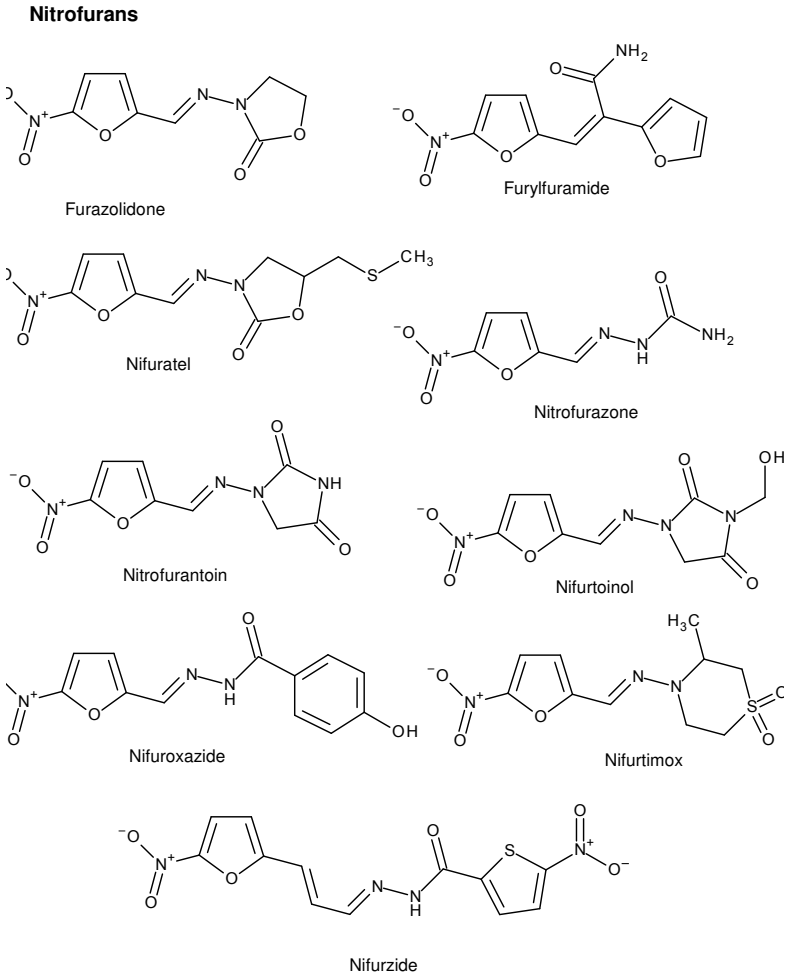

Figure 4: Nitrofuran drug substances

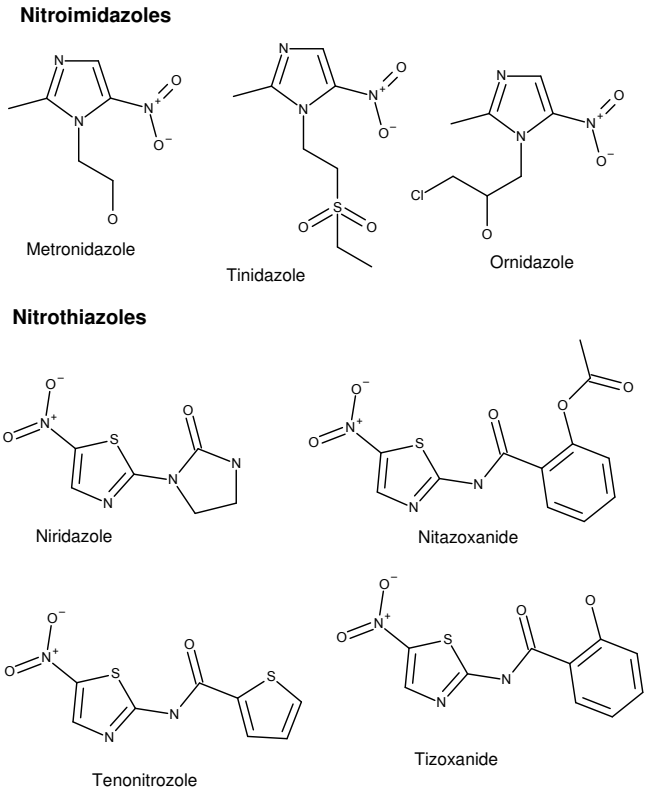

Figure 5: Nitroheterocyclic drug substances other than nitrofurans

product for the treatment of acute intestinal infections that is likely to be used for relatively trivial conditions.

Genotoxicity appears to be the mode of action of these products which, if I understand the situation correctly, are more toxic to microorganisms than to animals. The species specificity is attributed to the fact that the reactive metabolites rapidly become covalently bound to proteins and so, in eukaryotes, they don't readily migrate to the nucleus. This leaves open the question of potential immunogenicity; modified proteins can trigger an immune response, which may lead to allergic reactions to the product. Electrophiles of a certain size that react directly with DNA are, therefore, also potentially immunogenic, but this is a difficult subject that is not generally considered when discussing, for example, genotoxic impurities in pharmaceuticals.

Nitrofurans are not the only genotoxic drug substances approved for clinical indications other than cancer chemotherapy. They do, however, possess the distinction (at least in Europe) of being approved for human use although banned for the treatment of food animals [11]. Toxic residues accumulate in animal tissues because they are covalently bound to proteins, but when the meat is eaten they are released in the acidic environment of the stomach. Although it was stated [12] that nitrofurantoin is only weakly genotoxic in mice, there do not seem to be enough data for a quantitative estimation of the attributable cancer risk in humans; this situation of uncertainty appears to be common. Consequently (for the sake of argument), had these drug substances been discovered as impurities in some other drug substance, their daily dose would be limited to the default amount of 1.5 μg.

A known difficulty in evaluating weakly genotoxic chemicals is related to the need for unusually high doses to demonstrate an effect in animals. Under these conditions, nonspecific toxic effects, sometimes described as 'irritation', may lead cells to engage in increased gene expression or other nuclear activity. Consequently, DNA becomes relatively more exposed to attack by electrophiles, and linear extrapolation to doses below those that cause general toxic effects may thus lead to overestimation of the genotoxic effects of such low doses. It has been proposed that this is the case with chloroform, of which trace amounts are usually present in chlorinated drinking water. Conversely, the genotoxicity of nitro-aromatic antibiotics in eukaryotic models might be underestimated if the test system is not already infected by a relevant microorganism that induces an inflammatory reaction. This argument leads naturally to the following digression on intestinal infections and their treatment. It is important to understand that this proposition was written by someone who has not carried out research in the field:

3.0.1 Why isn't lower-intestinal cancer even more prevalant?

Normal commensal anaerobic bacteria in the lower intestinal tract use inorganic sulfate and other sulfur compounds as respiratory electron receptors. This results in the production of large quantities of hydrogen sulfide, a toxic compound that also has hormonal activity at low concentrations. Hydrogen sulfide from this source does not reach the general circulation because it is enzymatically oxidised by intestinal mucosal cells to the thiosulfate anion [13]. According to a recent paper, the inflammatory reaction induced by *Salmonella* Typhimurium infection is associated with oxidation of intestinal thiosulfate to tetrathionate (Figure 6), which the organism requires as a respiratory electron acceptor [14]. The pathological organism would not grow in this environment in the absence of tetrathionate. Now, commensal bacteria generate, in addition to H_2S, numerous potentially genotoxic electrophilic compounds [15]. Since the anionic sulfur function of thiosulfate is about the most reactive nucleophile known in aqueous solution, it could act as a scavenger for electrophiles that react via certain mechanisms (Appendix A.2.3). Infusions of thiosulfate have been used to protect non-target organs during cancer chemotherapy, although the effect is limited because the anion is rapidly eliminated and it does not enter cells. Also, the protective effect against nitrogen mustards would be limited for mechanistic reasons (Appendix A.2.4). This leads to the rather obvious speculation that depletion of thiosulfate might be part of the explanation for the suspected association between intestinal bacteria and intestinal cancers [15]. The idea is summarised for readers of different backgrounds in the annotated redox diagram shown in Figure 6. Analytical chemists are (were?) familiar with the oxidation of thiosulfate to tetrathionate by weak oxidising agents such as iodine. Somewhat less well known are iodimetric back-titration methods for alkylating agents such as the nitrogen mustards, which alkylate the S^- function (see reference [16]).

Whatever the outcome of future research on intestinal thiosulfate, it is clear that nifuroxazide should not be assumed to be safe on the basis of tests carried out in the absence of intestinal infection.

3.0.2 Could a genotoxic mode of action be a starting point for developing future antibiotics?

For the last 6 decades or so, antibiotics derived from microorganisms have been considered superior to the synthetic products, both for efficacy and for reduced frequency and severity of side-effects. However, drug resistance, which may be due in part to misuse of these products, is a major concern.

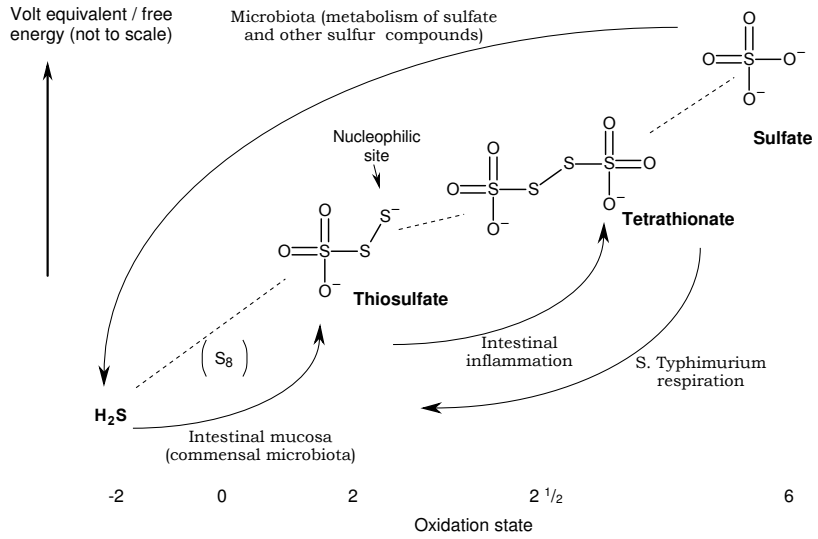

Figure 6: Transformations of sulfur species in the lower digestive tract.

If we can assume that medicinal chemists with little information on molecular drug targets had a "good eye" for effective products such as the nitroaromatics, it would make sense to speculate on how these leads could be further developed in the light of current knowledge and requirements. The idea of reactive metabolites that react with prokaryotic DNA but are sequestered before they reach the eukaryotic nucleus is appealing. In view of the recent epidemics of antibiotic-resistant microorganisms, a case could be made for developing new but perhaps not entirely safe products for use in life-threatening cases of infection, as is already the case with cytotoxic agents. The market for such products would be restricted, if only by the immunogenic risks, and the regulatory approach would have to be adapted accordingly. Calculated risks are taken with diagnostic ionising radiation in everyday medical practice; unfortunately it is not possible to calculate chemical risks with the same degree of accuracy.

4 Carbohydrate ethers

4.1 Manufacture, impurities and uses

Carbohydrate ethers were first developed in the 1920s and were soon widely commercialised. Major uses are as solvating or suspending agents, gelling

and thickening agents and adhesives. Since these products are ubiquitous, their safety should be kept under continuous review.

Quantitatively the major industrial products are cellulose ethers, though ethers of starch and of mono and disaccharides also exist. Common substituents are the lower alkyl groups, hydroxyethyl, 2-hydroxypropyl and carboxymethyl (usually as the sodium salt). Other derivatives including quaternary ammonium salts have been described. To illustrate the general structural features, methylcellulose and hydroxypropylmethylcellulose (HPMC) are illustrated in Figure 7.

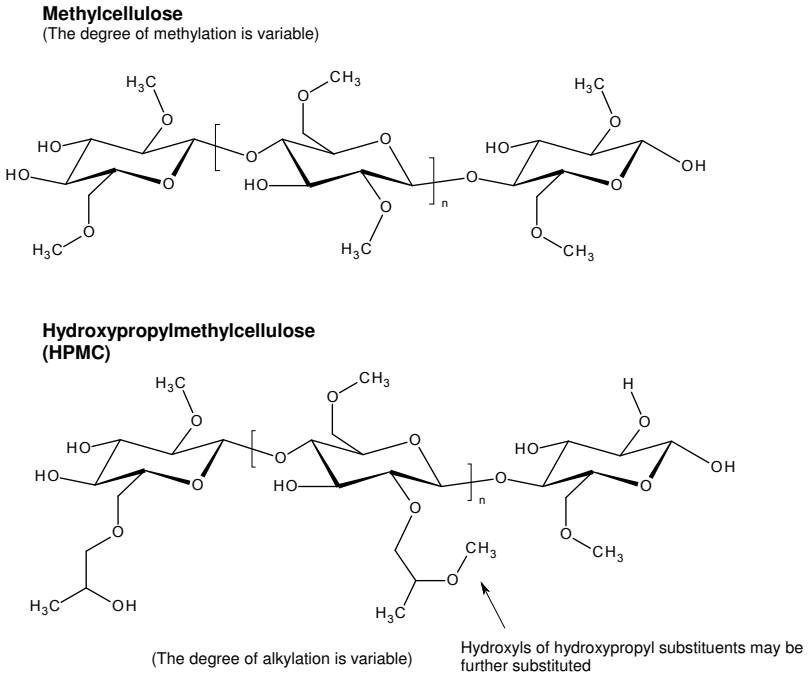

Figure 7: Methylcellulose and hydroxypropylmethylcellulose

Hydroxylated substituents may themselves be alkylated. Products with different properties are obtained by varying the molecular weight of the carbohydrate, the mix of substituents, and the degree of substitution. Some carbohydrate ethers, when of suitable quality, are approved as food additives, and are officially regarded on the basis of extensive experimental evidence as "Generally Recognised As Safe" (GRAS). Products in this class are extensively used as pharmaceutical excipients. I am aware that certain pressure groups are opposed to the use of excipients. However, without them it would be quite impossible to manufacture individual dosage units by the million, nor to ensure the bioavailablity of drug substances that are poorly soluble.

Carbohydrate ethers are manufactured using alkylating agents that are either known or strongly suspected to be direct-acting genotoxins. As mentioned in the introduction, testing for residues of these frequently-used reagents is required for drug substances, but has not been required for excipients (although this situation may be changing at the time of writing). The allowable dose of such genotoxic impurities in medicines is usually not more than 1.5 μg/day per impurity; presumably it should be lower than this for non essential food additives.

4.2 Chemical reactivity of carbohydrate ethers

4.2.1 Intrinsic reactivity

Simple aliphatic ethers have weak intrinsic acid-catalysed electrophilic alkylating activity. Carbohydrate ethers should be relatively more reactive because unsubstituted carbohydrates are already slightly acidic, and it could be speculated that neighbouring unsubstituted OH groups might have an activating effect, as with the *o*-alkoxy benzoic acids and amides (Section 2). I did not find any reference to this subject, which might be considered surprising in view of the widepread applications of the products. In the absence of specific information to the contrary, it would be prudent to suppose that a minority of substituents in some of these products might be exceptionally reactive, for steric or other reasons.

However, carbohydrate ethers are unlikely to enter cells, and so they would not be likely candidates as direct acting genotoxins. On the other hand, since they should be more reactive than simple ethers, it is likely that other more hazardous compounds could be formed by chemical interactions during manufacture, storage or use of formulated products containing them.

4.2.2 Reactions leading to formation of reactive alkylating agents in drug substances

A known and regulated hazard in pharmaceutical manufacturing is the formation of notoriously genotoxic alkylating agents by reactions between lower alcohols and approved drug substance counter-anions that have significant nucleophilic reactivity: notably sulfate, alkylsulfonate, arylsulfonate and halide ions. The alcohols are used in syntheses, as recrystallisation solvents, and as cleaning agents for manufacturing equipment; ethers, which are similarly reactive, do not seem to receive as much attention as alcohols. Chemistry textbooks discuss mainly acid-catalysed reactions, which involve protonation of the oxygen of the alcohol or ether. The chemistry is likely to be more

complicated, however, in the presence of drug substances. Salts of basic drug substances with strong acids sometimes have sufficient intrinsic acidity for reaction to occur, though the conditions necessary for this are unpredictable, particularly in the solid state. Solvent residues are often present in finished products and in such cases the concentration of reaction products may increase during long term storage.

This general type of hazard came to public attention in 2007, when tablets of nelfinavir (Viracept), a treatment for AIDS, had to be withdrawn urgently because the drug substance was contaminated with ethyl methanesulfonate (EMS). Residues of ethanol, used for cleaning manufacturing equipment, had come into contact with methanesulfonic acid, the salt-forming agent of nelfinavir. EMS is frequently used in the laboratory as a mutagen.

4.3 Possible formation of reactive alkylating agents in formulated drug products

In contrast to the case of drug substances and solvents, chemical interactions in formulated products seem to have received little attention. Whether or not a drug substance is acidic, solid dosage formulations are frequently made slightly acidic for reasons related to dissolution properties and physical, chemical and microbiological stability. The nucleophilic counter-anions listed above would, therefore, be expected to react to some extent with carbohydrate ethers used as excipients, to give haloalkanes or alkyl sulfate/sulfonate esters. These products are much more reactive than the ethers and are more likely to reach the cell nucleus. The kinds of reactions that could occur are illustrated below (Figure 8) for a hydroxyethyl carbohydrate derivative in the presence of a hydrochloride and a methanesulfonate (mesylate) salt.

Some drug substances are sulfate salts, which require a separate discussion. The sulfate dianion reacts with alcohols and ethers to form the corresponding monoalkyl sulfate anions, which are not usually recognised alkylating agents. Monoalkyl sulfates are salts of strong acids. The acids are known to disproportionate to sulfuric acid and the dialkylsulfate esters, which are reactive alkylating agents. The sequence of reactions that would occur in the presence of an excess of a strong acid is presented in Figure 9.

I did not find information on whether disproportionation of monoalkylsulfates has been observed under neutral or weakly acidic conditions, but this could be a possible explanation for sporadic reports that monoalkylsulfates do have some alkylating reactivity. The formation of dialkylsulfates, if it occurs, would be relevant to adhesives containing gypsum (calcium sulfate; see Section 4.4.2).

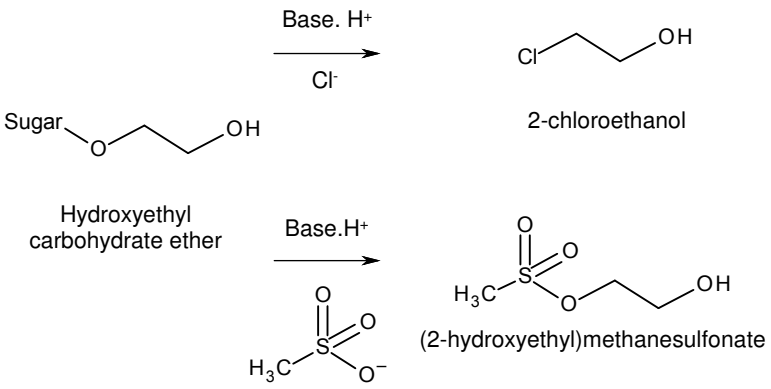

Figure 8: Reactions of carbohydrate ethers with counter-anions of salts of basic compounds

4.3.1 Notes on potential pharmaceutical hazards

Simple chlorocarbons, which would be formed from (hydro)chloride salts and unsubstituted alkyl ethers of carbohydrates, are established carcinogens, though they are at the low end of structure-activity correlations [2]. They are relatively weakly reactive alkylating agents but may form reactive metabolites. I have remarked [5] that traces of chloroethane are commonly found in drug substance hydrochlorides that have been recrystallised from ethanol. It should not be assumed that volatile degradation products such as this would be eliminated by evaporation; like volatile residual solvents, they are frequently strongly retained by adsorption. Surprisingly, chloroethane is also a compendial drug substance, despite its proven carcinogenicity; this is another example of a rather relaxed regulatory attitude towards genotoxic drug substances.

Hydroxyethyl carbohydrate ethers would react with chloride to give 2-chloroethanol (Figure 8), which provides an illustration of the uncertainties in this field: although this major industrial intermediate was expected to form essentially the same DNA adducts as the highly dangerous gas ethylene oxide, it took a lot of assiduous research to conclude that it is only very weakly carcinogenic. Apart from this example, alkylating agents with a

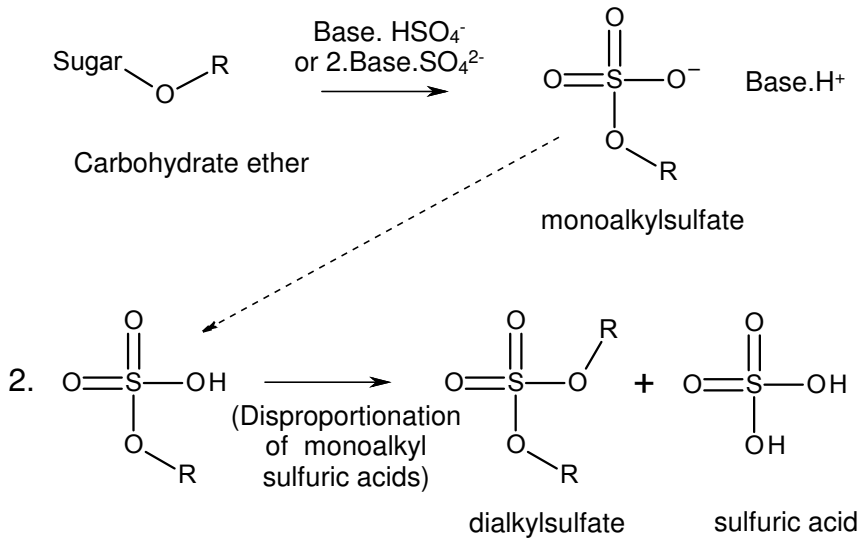

Figure 9: Formation of monoalkylsulfate from carbohydrate ether and sulfate salt, and possible disproportionation to dialkylsulfate

hydroxyl in the 2-position are considered to be more potent genotoxins than their unsubstituted analogues. Thus, 2-hydroxyethylmethylsulfonate, which (as illustrated in Figure 8) could be formed by reaction of a hydroxyethyl carbohydrate ether with a methanesulfonate (mesityl) salt, is useful as an experimental mutagen.

Many pharmaceuticals are (hydro)bromides, which should be of particular concern because bromide is generally a more powerful nucleophile than chloride, and bromoalkanes are more potent carcinogens than chloroalkanes. Furthermore, practically all chlorine compounds contain some bromine. The subject is not much discussed in the pharmaceutical literature because bromide is generally recognised as safe. Consequently, the control of bromide in chloride salts is rarely considered necessary. One exception is pharmaceutical-grade KCl, for which KBr is limited to 0.1 percent by weight. However, caution should be exercised when a chloride salt comes into contact with alcohols or ethers (including carbohydrate ethers), because of the relatively greater reactivity of bromide ion; the proportion of bromoalkane to chloroalkane would be expected to be greater than the initial bromide/chloride ratio.

Should the arguments presented above be considered worthy of concern regarding pharmaceutical formulations containing carbohydrate ethers, it

would be necessary to consider other nucleophiles such as monofluorophosphate in toothpastes.

4.4 Potential hazards of carbohydrate ethers in non pharmaceutical applications

4.4.1 Tobacco, and other applications involving pyrolysis

There are reports that tobacco smoke contains an unknown substance that ethylates DNA directly, that is, without need for metabolic activation. The regioselectivity indicates that the reaction mechanism is probably SN2. Apparently (and perhaps not surprisingly), resources have not been made available for the identification of the alkylating agent(s) responsible. Possible reasons for the predominant formation of ethylating agent(s) as pyrolysis product(s) are not discussed in the report I have seen [17]. It was not established whether the observation is specific to pyrolysis of this particular plant product, or whether it is associated with the manufacturing process. However, numerous additives are approved for tobacco, and one that is frequently mentioned is the adhesive ethylcellulose. It would be reasonable to speculate that, depending on the nucleophiles present in formulated tobacco, pyrolysis of this compound could be responsible for the ethylating reactivity. While there is a certain public tolerance of genotoxic pyrolysis products from natural sources such as food that has been fried or barbecued, this may not extend to the unknown pyrolysis products of semi-synthetic additives.

Some carbohydrate ethers are approved for use as food additives, though I did not find any applications that could be considered to provide major benefits, for example by enhancing conservation. Cooking could be described as a form of pyrolysis; possibly of concern may be the use of cellulose ethers as additives for batters for deep-fried food [18]. Here, the major nucleophilic anion concerned could be chloride (together with iodide from added salt).

Applications possibly involving workplace exposure to pyrolysis products of carbohydrate ethers may include surface coatings such as ceramic glazes.

4.4.2 Adhesives

A major use for carbohydrate ethers is in adhesives. Familiar domestic applications are in water-soluble glues and wallpaper paste. Large amounts are used in the building industry, in particular for plaster-based adhesives and renderings. Sometimes, all the internal walls and ceilings of a dwelling are rendered with these products. Since plaster (gypsum) is calcium sulfate, any possiblity that an interaction with cellulose ethers might yield dialkylsulfates

should be considered (Figure 9). The acid necessary for this interaction could be derived from oxidative or non-oxidative degradation of the carbohydrate and in case of fire by pyrolysis of the gypsum. Conditions to be considered would include overheating (proximity to radiators and heating or cooking applicances, plumbing work) and firefighting.

5 Summary and conclusion

Natural, synthetic and semi-synthetic chemicals may present genotoxic hazards that are not immediately obvious. The hazards considered here involve compounds or their metabolites that react directly with DNA, causing mutations that may have various effects including initiation of the sequence of events that leads to cancer. Three examples are presented that concern mainly but not exclusively pharmaceuticals.

The *ortho*-alkoxybenzamide series includes a number of valuable drug substances. However, all of these compounds have a somewhat obscure structural alert for genotoxicity which does not seem to have been investigated. This alert is based on chemical reactivities observed during stability studies and from information in a paper from the synthetic chemistry literature. The corresponding *ortho*-alkoxybenzoic acids are known impurities that are expected to be more reactive than the drug substances. Since the reported experimental conditions are not a reliable guide for predicting DNA adduct formation in vivo, the degree of any genotoxic risk remains to be evaluated.

Synthetic antibiotics of the nitroaromatic class are known to have genotoxic metabolites, and genotoxicity appears to be their mode of action. They are banned from administration to food animals but continue to be prescribed for administration to humans. This ambiguous situation may perhaps have inhibited research investment aimed at developing second-line solutions when antibiotic-resistant infections are life-threatening.

Carbohydrate ethers have numerous uses ranging from food additives through medicinal excipients to building materials. They are reputed to have negligible intrinsic toxicity. However, it is possible to envisage situations in which chemical interactions lead to the formation of highly reactive and potently genotoxic compounds.

The above list of potential hazards is by no means complete. I remember reading, a long time ago, a proposition that artificial detergents might promote cancers and other pathologies by insinuating themselves into lipid bilayer membranes. Furthermore, in the present context, cationic detergents of the alkyltrimethylammonium class are known alkylating agents (the reaction is used for analytical purposes). Sulfate esters, the most common

anionic detergents, are also suspected of having alkylating reactivity under certain conditions [19].

The risks, for individuals and for the population, associated with such potential genotoxicity are difficult to evaluate. Concerning the potential pharmaceutical hazards, the subject appears to fall outside the scope of research organisations in the field of pharmacovigilance.

Appendix A: Alkylation of DNA, and genotoxic endpoints

This appendix was intended initially to explain relevant aspects of DNA adduct formation to former scientific colleagues who were unfamiliar with the subject. I was surprised to be unable to find a suitable review, and discovered that a likely reason for this is a certain state of confusion in the primary literature.

Soon after the elucidation of the structure of DNA, researchers began to study the toxic effects of electrophilic reagents, particularly alkylating reagents, which can react with nucleophilic sites on the DNA molecule to form adducts (Figure 10). It had already been established, from work on chemical weapons and subsequently on chemotherapeutic agents, that such reagents are mutagenic and carcinogenic. The capacity of a reagent to form adducts *in vivo* depends not only on its reactivity and selectivity, but also on numerous toxicokinetic factors. The mutagenic consequences of adduct formation depend in turn in complex ways on the sites of the DNA adducts and on the structures of the substituent groups.

Mutations may be due to transcription errors affecting a single DNA base that has been chemically modified (Single Nucleotide Polymorphism; SNP), or to more radical damage that is not fully corrected by elaborate repair mechanisms that exist in all life forms. One possible consequence of a mutation is "intitiation" of the cell into a long and complicated sequence of events that result in a neoplasm. For those of us not involved in this field of research, it is sufficient to remark that organisms with differentiated cells and organs have incredibly complex means of restraint that ensure that cells can divide without proliferating uncontrollably in time or in space. Carcinogenic initiation can be caused by interference at the level of these control systems, which are downstream of DNA coding and transcription processes. Such effects do not concern us here because this document is limited to the chemical modification of DNA by electrophilic reagents.

Relating the chemistry of covalent reactions of direct-acting genotoxins with DNA to genotoxic endpoints such as cancer is a difficult interdisciplinary subject; a lack of accessible information hinders understanding of the kinds topics discussed here. For some purposes it may be sufficient to understand that compounds with certain types of chemical functional groups can act irreversibly with DNA, and that in some cases lifetime doses in the low milligram range may be significantly carcinogenic in humans. While exposure to traces of such compounds is, therefore, always cause for concern, the significance of any risk in any particular case is remarkably difficult to predict

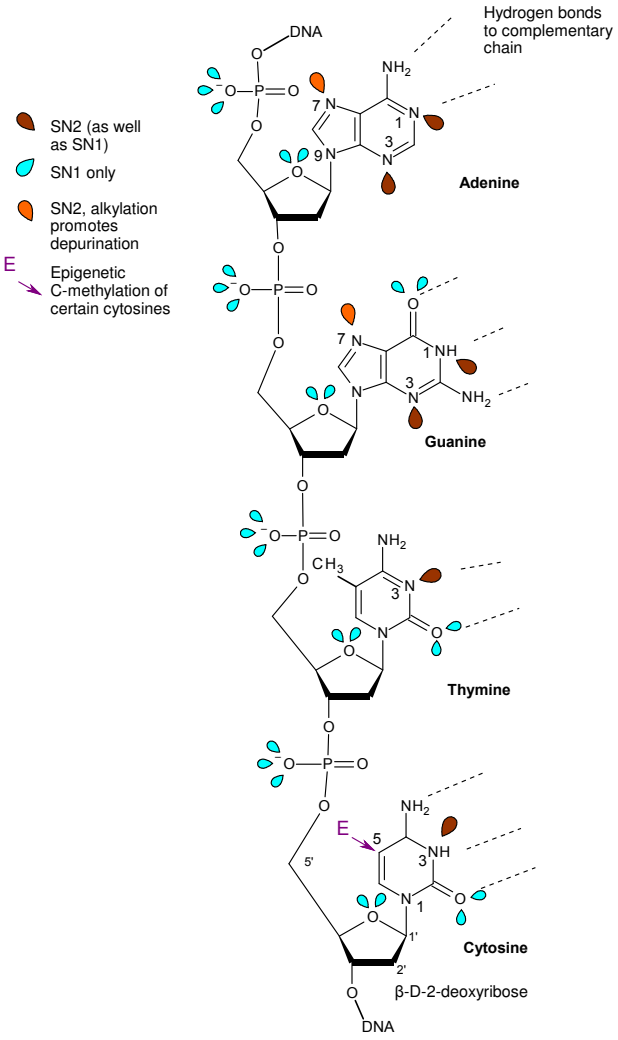

Figure 10: Sketch of major electrophilic sites on single-stranded DNA

or determine. A controversial aspect that will be discussed only with respect to one relatively neglected point (Appendix A.2.3), is the shape of dose-response curves for carcinogenicity, taking into account that an individual is exposed simultaneously to low concentrations of numerous electrophilic compounds. Statistically significant responses can only be observed at high doses because of the high and variable natural incidence of cancer. The authorities continue to require that observed responses are to be extrapolated linearly (hence conservatively) through the origin (zero dose/zero response). Particular cases of threshold-type (sigmoid) responses have been published, but it is not yet known if general conclusions can be drawn from these studies.

The literature on correlations between chemical structure, reactivity and mutagenesis is confusing even for readers familiar with it. A recent critical review of one aspect of this subject [20] notes some 'oversimplistic' approaches and remarks on the absence of a general review covering the key aspects. My purpose in writing this appendix is partly to provide some background information, but also to add to the above criticism [20]; a reader new to the subject might doubt the validity of much that has been written.

A consequence of this scientific confusion is that, as a specialist with limited wider knowledge of the subject, I am unable to decide to decide whether any of the potential hazards I describe are in fact worthy of discussion.

Appendix A.1: DNA adduct formation by electrophilic reagents

Electrophilic reagents interact with electron-rich (nucleophilic) sites which, in the present context, consist of lone pairs of electrons on DNA heteroatoms[2] oxygen and nitrogen. Different electrophilic reagents have different selectivities for these heteroatoms. The genotoxic consequences of formation of a DNA adduct depend on the position as well as the nature of the adduct.

Lone pairs are electrons of the outer orbital shell that are not directly involved in chemical bonding but can interact with positively charged or polarisable reagents. The quantum mechanical probability density functions of these electrons can be sketched, as shown in Figure 10 for a single strand of DNA. The different 'kinds' of lone pair (depending on the atoms and their situation in the rest of the molecule) have different quantum mechanical properties and interact differently with different kinds of electrophilic reagents. The lone pairs shown in blue in Figure 10 are not very polarisable and they

[2]In organic chemistry a heteroatom in a chain or ring of carbon atoms is any atom other than carbon or hydrogen. Some of these have significant electron density in lone pairs. Consequently, other atoms in the molecule (including carbon) are positively polarised.

interact only with species with regions of high charge density, generating high electric fields. The others (some nitrogen atoms) have, in addition, specific kinds of interaction with certain kinds of reagents that are not necessarily very reactive intrinsically.

These differences in reactivity are described in terms of chemical reaction kinetics and mechanisms which, ultimately, can only be understood in terms of quantum mechanics. Individual molecular events occur on the timescale of a single molecular vibration, of the order of a picosecond or less. Only recently has it become possible to elucidate reaction mechanisms directly, using a combination of femtosecond spectroscopy and quantum mechanical molecular orbital calculations. Understanding at the practical level (synthetic chemistry, toxicology) has been the result of the systematisation of laboratory observations, guided by quantum mechanical concepts. Semi-empirical correlations, particularly that of Swain and Scott [21], have been of some use in understanding DNA damage mechanisms but, since they conceal much of the complexity, they do not apply to all situations and are frequently misinterpreted. Research into this aspect of genotoxicity has been characterised by failures to understand the underlying chemistry, a situation aggravated by an unfortunate choice of experimental models.

We confine this discussion to alkylation reactions, sometimes termed 'nucleophilic substitution at a saturated carbon atom', and attempt to clear up possible confusion about the ways the reactions can be described. Free radical reagents (compounds with unpaired electrons and hence magnetic fields) react almost indiscriminately and they are toxicologically significant; however, we can neglect them here. Observations on substitution reactions in which electric fields are involved can be described in two ways which are not mutually exclusive but which have led to terminological confusion in areas outside the remit of organic chemistry textbooks:

- A usually valid generalisation is that the more reactive the electrophilic species (genotoxic reagent), the less selective will it be with respect to the targets it reacts with (lone pair-bearing DNA heteroatoms). No reaction mechanism is implied here, but there has been a tendency to confuse rapidity with the SN1 mechanism, whether or not the kinetic or other parameters are consistent with this. The classical definition of an SN1 reaction is confined to cases such as reaction 2 in Figure 16, in which a 3-coordinate carbonium ion is formed. The alkyldiazonium ions formed from nitrosoureas (reaction 4 in Figure 16) are sufficiently reactive to be unselective, but this is not an SN1 reaction.

- In some cases, the detailed reaction mechanism is known, and this should be of value for studies of genotoxicity. Examples are reactions

1-3 in Figure 16. It is known that reaction 4 involves initial formation of the methyldiazonium ion, but the second step does not seem to have been fully explored. "Pure" SN1 reactions (reaction 2) tend to be unselective. The two-stage SN2 reaction (3) is important because this is the mechanism of the nitrogen mustard cytostatic agents. Unfortunately, some authors have mistakenly considered it to be SN1 because the rate of disappearance of the starting compound (see below) is independent of that of the nucleophilic substrate. Later (Appendix A.2.4), we will discuss a particular toxicokinetic property of two-stage SN2 reactions that appears to be ignored in the recent literature.

Appendix A.1.1: SN1 and other non-selective reactions

In physiological media, the most reactive (hence unselective) species being considered must always be formed by a slow initial step *in situ* because they react practically instantaneously with water[3]. The most-studied "classical" reaction of this kind is termed 'SN1' (substitution, nucleophilic, rate determining step (the first step here) unimolecular). As mentioned, the highly reactive species has a positively charged carbon atom (carbonium ion).

Figure 11 shows the sequence of events in SN1 reactions of a bromoalkane. The bromine atom could be replaced by another electron-withdrawing entity such as an alkylsulfate radical without changing the argument. When dissolved in a suitable solvent (sufficiently polar to enable charge separation), molecules of some bromoalkanes dissociate spontaneously at random intervals to give the bromide ion; the rate depends only on the temperature and the solvent. A positive charge is left on the carbon atom to which the bromine had been bound. This carbonium ion will combine essentially instantaneously with the first lone pair of electrons it collides with, to form a covalent bond. If no other lone-pair-carrying molecule is present, it will react with bromide to re-form the original molecule (not shown). If water is present (Figure 11), the carbonium ion will react at essentially every collision with one of the oxygen lone pairs to give the corresponding alcohol. We also show in Figure 11 the reaction with a tertiary amine to form a quaternary ammonium bromide. If the molar concentrations of amine and water are the same, the ratio of products (quaternary amine : alcohol) will be approximately unity.

A "true" SN1 reaction is unselective because the carbonium ion is too

[3]One motivation for writing this section is a case in which an analyst working for a pharmaceutical company was expected to determine trace residues of a synthetic reagent in a drug substance. This could not be done because the reagent reacts so rapidly with traces of water that suitably dilute solutions could not be prepared; the compound had tested positive for genotoxicity because of an artefact of the test methods.

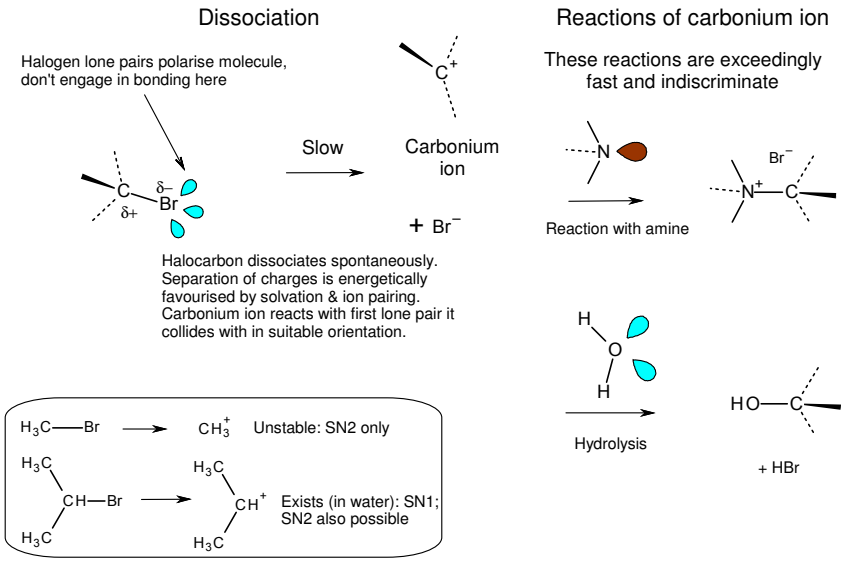

Figure 11: SN1 reaction mechanism

reactive to distinguish between tightly-bound "hard" oxygen lone pairs and the relatively "soft" (diffuse) nitrogen lone pairs; it forms a new molecular orbital with the first lone pair it bumps into (typically within tens or hundreds of picoseconds). We recall that DNA that has been alkylated on oxygen is relatively less effectively repaired.

We ought to mention that his simplified description of the SN1 mechanism neglects the fact that complete separation of bare electric charges in solution just can't happen; too much energy would be required. In reality, the energy of separation between the carbonium and bromide ions in Figure 11 is dissipated by attractive forces between the ions and polarised regions of polar solvents, by ion pairing, and by the effect of a solvent with high dielectric constant. Water is a particularly effective solvent in these respects, though its reactivity can complicate investigations. The carbonium ion forms part of a non-covalent molecular cluster. The bromide or other anion ('leaving group') also needs stabilising, but less energy is involved for 'good' leaving groups such as this. Since the largely (but not entirely) aqueous environment of eukaryotic DNA in its different states is not well understood, reaction mechanisms *in vivo* may sometimes be in doubt.

Appendix A.1.2: SN2 reactions

Certain substituted alkanes can never form a carbonium ion cluster having a lifetime in solution anywhere near the time interval between molecular collisions. The simplest case is the methyl carbonium ion CH_3^+. If we dissolve bromomethane (or methylmethanesulfonate) in water at room temperature, it will react by hydrolysis to form methanol only very slowly, because there is no dissociation (SN1 reaction), and for quantum mechanical reasons the intact molecule does not interact rapidly in the SN2 manner with the oxygen lone pairs of water. If now we add an amine to the aqueous solution, bromomethane will react relatively rapidly with it (Figure 12), at a rate that is proportional to both the amine and the bromomethane concentrations. Note that if the reaction were SN1, the amine would not affect the rate of disappearance of the bromomethane unless its concentration were high enough to affect the properties of the solvent. Spectroscopic observations and quantum mechanical calculations confirm earlier deductions that bromomethane and the lone pair of an amine can form a neutral 'transition state' structure of sufficiently low energy for it to exist at least on the timescale of atomic movements (picoseconds). This structure, with 5-coordinated carbon, can then separate into the original molecules or into the alkylated amine and bromide ion (Figure 12). Bonding of the "soft" nitrogen lone pairs to bromomethane is energetically more favourable than of the "hard" oxygen lone pairs. Consequently, "SN2" electrophilic reagents like bromomethane react more readily with certain nitrogen atoms of DNA than with the oxygen atoms (Figure 10).

The terms "hard" and "soft" were introduced during the 1960s as an empirical way of rationalising observed reactivities without delving too deeply into quantum mechanics. They may perhaps have outlived their usefulness but may help in picturing the different reactivities of nuclophilic sites on DNA.

Appendix A.1.3: Mixed SN1/SN2 mechanisms

According to the simplified descriptions above, a "pure" SN2 reaction mechanism would lead exclusively to the alkylation of certain nitrogens of DNA, and a "pure" SN1 mechanism would result in indiscriminate alkylation of oxygen and nitrogen. In practice the selectivity (described by the Swain-Scott s parameter) varies continuously. Individual molecular encounters might in a particular case be SN1 or SN2, or else the detailed events on the picosecond timescale constitute some kind of hybrid mechanism the discussion of which is best left to the physical organic chemical fraternity.

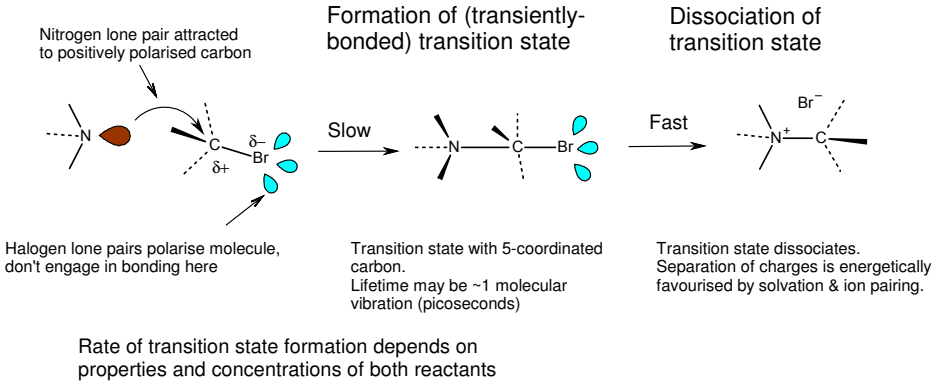

Figure 12: SN2 reaction of bromomethane with an amine

Ethylmethylsulfonate (EMS) is often presented as an SN2 reagent [7], but it is slightly less selective than methylmethanesulfonate (MMS), which can only engage in SN2 reactions. We inadvertently provided a graphic demonstration of structure-dependent selectivities when we developed an analytical method for these reagents, together with the "pure" SN1 reagent *iso*propylmethanesulfonate (iPMS). Since the direct determination of traces of these polar and reactive compounds is difficult, we prepared more amenable derivatives by reaction with aqueous sodium thiocyanate, Na^+NCS^-, and determined the products by gas chromatography. The thiocyanate anion interests physical organic chemists because it is bifunctional: "SN2" reagents react mostly at the "soft" sulfur end to form alkylthiocyanates RSCN, and "SN1" reagents react at both ends to form about equal amounts of alkylthiocyanates and alkylisothiocyanates (RNCS). In Figure 13, it can be seen that MMS forms MeSCN with only a trace of MeNCS, whereas iPMS forms about equal amounts of iPSCN and iPNCS. EMS is intermediate; it forms a rather larger proportion of the "SN1" product (EtNCS) than does MMS.

Appendix A.1.4: Notes on DNA reaction sites

Some heteroatoms such as exocyclic nitrogens are not major sites of DNA alkylation.

For readers unfamiliar with the kind of tautomerism that fooled Watson and Crick for a time, we recall that the carbonyl oxygens (C=O) give the ethers of the corresponding enols (Figure 14).

Adducts formed by SN2 reactions (specific for some ring nitrogen sites) are relatively effectively removed by DNA repair mechanisms. When an

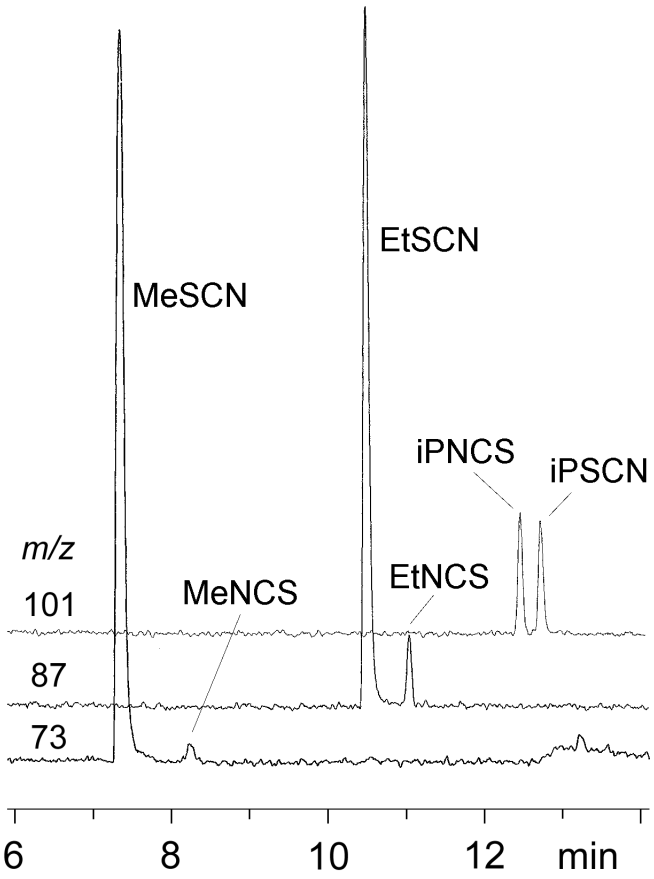

Figure 13: Chromatogram of alkyl thiocyanate/isothiocyanate derivatives formed by reaction of the corresponding alkylmethylsulfonates with aqueous thiocyanate (NCS^-). The alkylthiocyanate (RSCN) derivatives are formed preferentially by the SN2 reaction mechanism, whereas SN1 reagents form similar amounts of RSCN and the isomeric isothiocyanates RNCS. Gas chromatography-mass spectrometry (electron ionisation), with recording of the molecular ions of the derivatives.

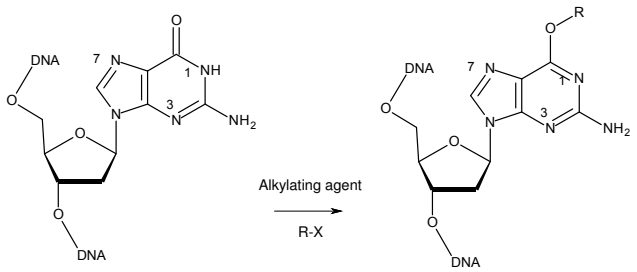

Figure 14: Alkylation of the carbonyl oxygen of a DNA base, forming the ether of the enol tautomer

adduct escapes repair, the most intuitively obvious genotoxic effects are misreading or failure to read the affected base, either during gene transcription or in actions concerned with the regulation of gene expression. Depurination and strand breakage are more drastic forms of damage that are not explained each time they are mentioned in review articles. These reactions occur when adenosine and guanosine bases are alkylated at the 7-position as shown in Figure 15. Strand breakage can be repaired, but it frequently leads to mutation.

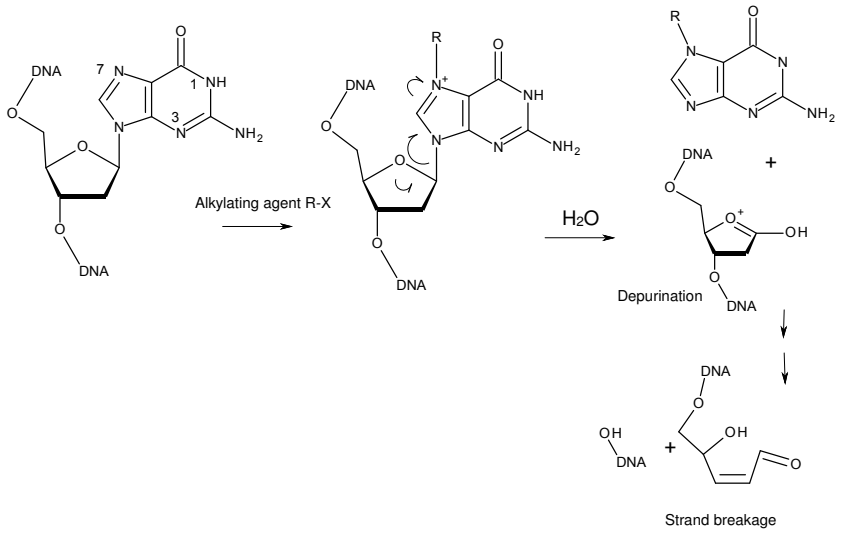

Figure 15: Depurination, possibly followed by strand breakage, when purine bases are alkylated at the 7-position

We recall that the (geno)toxicologist is interested in whether an electrophilic reagent reacts preferentially with certain DNA nitrogens, or indiscriminately (both nitrogen and oxygen adduct formation). Selectivity for nitrogen generally indicates the SN2 mechanism. This is not always understood by authors who confuse reaction kinetics with reaction mechanism. An unfortunate consequence was a controversy concerning the clinically important nitrogen mustards, of which a "half-mustard" analogue is shown in reaction 3 of Figure 16. Although the second step of the reaction is definitely SN2, the anti-cancer agents are potently genotoxic. The explanation that has been given is that the most effective agents have two functional groups and act by crosslinking the complementary strands of DNA. Another likely expla-

nation that tends to be neglected is that, because of the slow initial reaction to form the aziridinium ions, these products will partly escape scavenging by reactive endogenous nucleophiles (Appendix A.2.3).

Appendix A.2: Predicting and evaluating reactivity and selectivity of DNA alkylation reactions

Until recently, evaluating the kinetics and selectivity of DNA adduct formation directly by analysing the modified DNA was a difficult and expensive exercise. The latest techniques using liquid chromatography-mass spectrometry are of great value, but they remain expensive. Ideally, studies on mutagenicity by 'direct-acting' mutagens should include data on the extent of DNA modification under the experimental conditions that were used, because of the experimental and toxicokinetic uncertainties associated with reactive test compounds. Unfortunately, such controls would add greatly to the cost and complexity of each study.

Historically, purely chemical systems have been used as surrogates for DNA. The reagents were developed initially for analytical purposes, notably for the detection and determination of chemical weapons. Early methods were usually colorimetric, partly because only simple instrumentation is required. The most popular reagent is 4-(4-nitrobenzyl)pyridine (NBP), and it has been proposed in a recent critical review that it could still be of value [20]. The principle of this test method is given in Figure 17. Alkylation (or acylation) of the pyridine nitrogen yields a colourless pyridinium ion, that forms a coloured tetrahydropyridine upon addition of base. Frequently, the initial reaction is carried out in the presence of water, and the tetrahydropyridine is extracted into an organic solvent. Since the visible spectra of similar derivatives do not vary much, it is possible to evaluated different electrophiles approximately, without performing a calibration for each. NBP has been used extensively, particularly when the large number of electrophiles to be studied prevents the application of traditional chemical kinetic techniques.

The SN2 nucleophilic reactivity of NBP is reported to be about equivalent to the N7 nitrogen of the DNA base guanosine. Selectivity studies are usually carried out using competition between nucleophiles that have different properties. However, there is no generally-accepted reagent, complementary to NBP, that reacts also with 'SN1' or other highly reactive and unselective species. A widely-adopted approach is to carry out studies in buffered solutions containing water. Selective (SN2) electrophiles will react only with NBP, giving a high yield of coloured product, whereas less selective ones will react also with the water (and/or the various buffers that have been used) to an extent that reflects their lack of selectivity (Figure 18).

The review cited above [20] mentions several inappropriate or misinterpreted applications of the NBP method. In the following section, I take these comments further, and remark on a lack of innovation that seems to indicate

that not all researchers in the field have been aware of the severe limitations of the approach.

Appendix A.2.1: Deficiencies of the NBP reagent

Unless the reagent is freshly purified (or purified and then stored appropriately), the blank value is high, and the colour fades inconveniently rapidly. This is a serious cause of variability, though not all reports indicate whether purification was carried out. In earlier times, the purification of reagents and solvents was a common laboratory practice; this has mostly died out, and pure reagent does not seem to be commercially available.

Even with pure NBP, the coloured tetrahydropyridine derivative is not stable in the presence of air and water. Some authors take steps to exclude air [22], though total exclusion can be surprisingly difficult; see also Appendix A.2.2. The colour is stable if the reaction is done in the presence of a cyclodextrin that traps the derivative in its lipophilic interior; this also obviates the need for solvent extraction but may affect the response to different R-groups [23]. Another approach is to separate the initially-formed pyridinium cation by chromatography before developing the colour by addition of base. Several methods using thin layer chromatography have been described. We used liquid chromatography with post-column addition of the base (unpublished; see [16]). This method was surprisingly sensitive and easy to develop; the only disadvantage is that post-column reaction detection is not popular in pharmaceutical analytical circles[4].

A major difficulty for biological applications of NBP is its poor solubility in water. An organic co-solvent is usually required, and this affects reaction kinetics and possibly the reaction mechanism. According to our own work, *iso*propylmethane sulfonate has a half life in water of less than an hour at ambient temperature (about 21 °C)[24], but the half life is 35 h at 35 °C in 60% acetone, a frequently-used solvent mixture [22].

It has been proposed that the dielectric constant of aqueous-organic solvents that are used with NBP is similar to that of the major groove of DNA (see [20]). This apologetic argument highlights, in fact, a serious limitation of any technique in which the nucleophilic model is other than intact DNA in the natural environment of cells that are in relevant states of activity. In quiescent segments of a chromosome, the DNA is supported and shielded by specific proteins. It is thought that it is not very vulnerable to electrophilic

[4]Analysts working in this field tend to prefer standard techniques and off-the-shelf instrumentation that is in regular use. The main obstacle to using a wider variety of well known techniques is the time and expense of exhaustively qualifying instruments and validating methods according to rigid guidelines.

attack in this state, though this may depend on steric factors and reaction mechanisms. Unshielded DNA is more vulnerable, and when the two strands are unwound, steric constraints are further reduced and the heteroatoms involved in chain linkage by hydrogen bonding (dotted lines in Figure 10) become more accessible. The local polarity of the nuclear medium will obviously vary under these different conditions, and will be difficult to establish. Normal cellular activities include DNA maintenance and repair, regulation of gene expression, the process of gene expression, and cellular replication. These activities may be increased under some pathological conditions, and therefore studies on healthy organisms may not necessarily reflect genotoxicity in all conditions of real life.

In view of the extensive applications of NBP, it is surprising that a water-soluble and if possible more stable replacement has not been proposed, bearing in mind also that the compound has an obvious structural alert for genotoxicity.

Appendix A.2.2: Choice of reaction co-solvent

I hesitated to write this section, because it is difficult to believe that so many blunders could have been made in such an important field.

NBP was introduced as a chemical analytical reagent. Particular co-solvents will have been chosen for various reasons, including the possibility of enhancing reactivity. A criterion not often mentioned is the availability of solvents sufficiently pure to give low blank absorbance. Acetone is the most frequently used, but it presents a hazard if the desired reaction temperature is higher than the boiling point. Most often, the higher-boiling replacement is methyl ethyl ketone or benzophenone, though I was unable to obtain off-the-shelf supplies of suitable quality. I have successfully used a grade of 2-methoxyethanol that was marketed for the Moore and Stein amino acid analysis technique. It is no longer available because of toxicity for the reproductive system.

A colleague once proposed that ketones may be the preferred solvents for analytical purposes because they would catalyse the alkylation of the pyridine nitrogen by a mechanism involving the well-known reversible reaction involving attack of the tertiary amine on the carbonyl carbon. I do not remember the details of the chemical 'curly arrows', but it seems possible that in the presence of a ketone the initial step in the alkylation of NBP could involve alkylation of a negatively-charged oxygen. This would hardly be a good surrogate for N7 of guanosine.

Ketones having an alpha hydrogen present an additional difficulty that has not being noticed by researchers in the field of genotoxicity, although

it had previously been described in the analytical chemistry literature [25]. Reference and test alkylating agents are frequently iodoalkanes (Figure 19) or bromoalkanes. In such cases alkylation of NBP (I) yields one equivalent of the halide anion and the alkylpyridinium cation II. If the halide is bromide or iodide, and any oxygen is present, it is oxidised to the element. Bromine and iodine rapidly replace ketonic alpha-hydrogens to form the alpha-haloketones, which are reactive alkylating agents. In the example shown (Figure 19), iodoacetone reacts with a further molecule of NBP to form III which, when base is added, gives the coloured product IV. Formation of III recycles the iodine atom as iodide, resulting in amplification of the colour reaction. Since the reference compound defined by Swain and Scott [21] for reactivity and selectivity studies is bromomethane, and haloalkanes are frequently used as test compounds, any such studies involving a ketone with an alpha-hydrogen should be considered with caution.

Appendix A.2.3: Influence of reaction mechanism and presence of scavengers on shape of dose response curve

The linearity or not of genotoxicity dose responses is the subject of much research, some of it understandably industry-sponsored. I will discuss this only as far as is necessary to point out an aspect that seems obvious but is neglected in the recent literature that is available to me.

Regulatory authorities assume that, for a given kind of damage to DNA, the risk of cancer or other endpoint is directly proportional to the number of damaging molecular events. Consequently, the risk is directly proportional to the dose. This has been fairly well established for ionising radiation [26], a conclusion that is likely to be confirmed by ongoing studies on patients subjected during recent decades to the significantly high doses used in x-ray tomography[5]. Exposure of DNA *in vivo* to genotoxins at doses entailing the level of risk encountered during normal life are relatively much more difficult to evaluate than is the case with ionising radiation. For this and other reasons, studies of chemical mutagenicity and carcinogenicity have to be carried out at large doses, and it is widely believed (at least in the chemical and pharmaceutical industries) that the required linear extrapolation to more realistic doses is unduly conservative, with serious economic consequences.

There are several reports that certain electrophilic reagents, but not others, have a threshold-type response (similar to the start of a sigmoid re-

[5]A small proportion of the damage due to diagnostic x-rays is due to direct hits by photons on DNA; most of the effect is ascribed to reactive molecules formed by radiolysis of surrounding metabolites. Consequently, there is much in common between radiation damage and genotoxicity.

sponse) in certain experimental models. One such study, of mutagenesis in a cultured human lymphoblastoid cell line [7], will be described because it was followed up by another one on the induction of a DNA repair mechanism [27]. The compounds studied were the methylating agents methylmethanesulfonate (MMS) and methylnitrosourea (MNU), and the ethylating agents ethylmethanesulfonate (EMS) and ethylnitrosourea (ENU). The two alkylsulfonates showed a threshold below which there was no mutagenic response, a result consistent with the sigmoid response seen in many toxicological situations. The nitrosoureas had no threshold and the response was approximately linear. The difference between the two classes of reagents was ascribed to differences between the sites of adduct formation on DNA. MMS reacts exclusively by the SN2 mechanism (reaction 1, Figure 16) and only certain ring nitrogens of DNA are alkylated (Figure 10). EMS, which was more extensively studied, is similar, though the mechanism is less exclusively SN2 (Appendix A.1.3). The two nitrosoureas react via alkyldiazonium ions that are so reactive that the reaction is non selective and can be considered for practical (though not theoretical) purposes to be "SN1-like" (reaction 4, Figure 16). Thus, it is possible to compare the effects of selective and unselective alkylations involving identical small alkyl groups.

It has long been considered that the damage caused by predominantly SN2 reagents like MMS as EMS is effectively repaired by one or more of the known DNA repair mechanisms, and such activity was indeed demonstrated for EMS [27] under the conditions of the genotoxicity study [7]. There was no repair activity in response to the nitrosoureas, which is consistent with the observation that, for a given R-group, unselective reagents are relatively more mutagenic.

To summarise, it was demonstrated directly that in a human cell line SN1-like alkylating agents induced mutations as the expected linear function of dose, whereas the response to (predominantly) SN2 reagents showed a threshold below which there was no effect. The difference was ascribed to the greater efficacity of DNA repair mechanisms for SN2 (ring nitrogen) DNA alkylation adducts. While a complete analysis would have required simultaneous assessment of the extent and specificity of DNA alkylation, such data were, quite understandably, not available.

Although these authors [27] have demonstrated that DNA repair mechanisms are indeed induced specifically by SN2 reagents, the results presented do not prove that the observed response threshold was due entirely to DNA repair. Several possible explanations for threshold effects are given in another publication [6], and possible shapes for dose-response curves in the presence of DNA repair have been simulated [28]. A known additional explanation was discussed in 1981 by Spears [29]: the selective and saturable scaveng-

ing action of endogenous metabolites. The existence of this mechanism is implicit in the conduct of substrate competition studies used to establish nucleophilic specificities [29], but seems to have been forgotten in the recent literature. Scavenging may be relevant to some confusion over the mode of action of the classical nitrogen mustard anticancer drugs (see below). The omission seems surprising because the information could further allay fears about human exposure to certain industrial alkylating agents such as those used in the synthesis of carbohydrate ethers (Section 4), which could possibly be impurities or degradation products. To judge from declared sources of finance of a major research group [6], [7], it can be assumed that such reassurance is one of the objectives of current fundamental research in this area.

A drug safety alert in 2007, in which the AIDS drug nelfinavir mesylate was found to be contaminated with ethylmethylsulfonate (EMS), was considered not to be critical on the grounds that DNA repair mechanisms are effective for this compound; the additional argument involving selective scavenging was not mentioned in the review I was able to obtain [30].

The DNA of eukaryotic cells is protected from extraneous influences by various barriers including the cytoplasm and the nuclear membrane. These elements would be expected to contain endogenous nucleophilic sites or molecules that should scavenge electrophilic compounds, including normal metabolites. The "SN2" nitrogen sites of DNA (Figure 10) are less reactive than some likely scavenging endogenous nucleophiles. Powerful nucleophiles that are frequently mentioned in other contexts include glutathione and S-adenosylcysteine, the reaction product of S-adenosylmethionine (see Section 2.1). I have speculated on the possibility that thiosulfate might have a similar role in the gut epithelium (Section 3.0.1). To a reasonable approximation, such scavenging would be effective mainly against classical SN2 reagents, since a highly reactive nucleophile would have no more effect than water at removing a classical SN1 reagent. It is easy to show, even with a single-compartment model, that a low (saturable) endogenous concentration of a reactive scavenger would give rise to a threshold-type dose response to an SN2 reagent. Expressed qualitatively, a pulse dose of methylmethanesulfonate (MMS) (Figure 16) or ethylmethanesulfonate (EMS) would be destroyed by the above-mentioned sulfur nucleophiles before the compound could react with DNA, provided the dose is small enough. By contrast, molecules of *iso*propylmethanesulfonate (iPMS) in aqueous solution dissociate to the carbonium ion at random times that are unaffected by any nucleophiles present. The carbonium ion reacts with the first nucleophile it collides with (within picoseconds); any molecule of iPMS that dissociates close to a strand of DNA is likely react with it, a process that is practically unaffected by the presence

of scavenging nucleophiles. As explained earlier, the nitrosoureas MNU and ENU can be considered to react like iPMS.

This proposal of endogenous scavenging nucleophiles should raise further questions, for example about a role in handling the continuous normal exposure to genotoxins (including acylating agents), and whether metabolites exist specifically for this protective purpose. It seems likely that most nucleophiles to which people are exposed would be of the SN2 variety, since ubiquitous traces of water would be expected to degrade classical SN1 agents.

The argument is a little more complicated for the nitrogen mustard anticancer agents, and in view of occasional confusion in the literature, these important drugs will be discussed in the following section.

In conclusion, DNA repair and scavenging by endogenous nucleophiles are complementary protective mechanisms. There exist several DNA repair mechanisms, highly conserved across species. Each of these is more or less error-prone, and their efficacy depends on the nature of the DNA adduct; these factors could be a source of uncertainty in a regulatory context. DNA-methyltransferases are inducible, and they reliably reverse alkylation reactions where the last or only step is of the classical SN2 type. However, they are specific to a few small alkyl groups such as those used for the studies cited above [27]. Scavenging by endogenous nucleophiles should be effective for eliminating low doses of reactive electrophiles that react in a single step by an SN2-like mechanism, regardless of the nature of the DNA adduct. They would be less effective with two-step reactions such as those of nitrogen mustards (Section A.2.4), and with high doses of weakly-reactive SN2 reagents (Section 2).

Appendix A.2.4: Mode of action of nitrogen mustard anticancer agents

We recall that the nitrogen mustard anticancer agents were developed, long before Watson and Crick, as chemical weapons and subsequently as cytotoxic anticancer agents. They act by reacting at nucleophilic sites on DNA. The first agent approved for clinical use (in 1949) was mustine (Figure 21), a bifunctional agent that can introduce cross-linking within the double helix. The clinical interest of compounds of this class is that, although they are potently mutagenic and carcinogenic (2-3 orders of magnitude greater than monofunctional homologues), the ratio of cell death (the required effect) to mutation is relatively favourable. Some early misunderstandings [31] about the nature of the two steps of the alkylation reaction seem to have persisted over the years. It is now well established that the second step is SN2 (selective for nitrogen) and that the high potency is due to what appears to be a low

but highly toxic incidence of DNA crosslinking.

To simplify the discussion and remain on ground with which I am familiar, I will discuss first a "half mustard" of mustine, for which the usual trivial name is N,N-dimethylaminoethyl chloride (DMC; Figure 20). DMC is an intermediate in the synthesis of some drug substances. As it is a known carcinogen, though less potent than mustine, questions have been asked about possible residues in medicines since (to my knowledge) the early 1980s. We were called upon to develop an analytical method [16] following one such safety alert [3] that might perhaps have raised questions about the decision-making processes within a regulatory authority.

DMC and mustine are usually supplied as the hydrochloride salts, and they are quite unreactive when protonated, for example in acidified aqueous solution. The chlorine atoms are not readily amenable to direct intermolecular nucleophilic substitution under physiological conditions. The free bases are also quite stable in non-polar solvents and in the gas phase, a property that has been used for analytical purposes [16]. However, when the free bases are in aqueous solution, a reaction involving charge separation is thermodynamically favoured by the polar solvent: an internal nucleophilic substitution yields the cyclic aziridinium anion (Figure 20) and the chloride cation (not shown). DMC is about 50 % in the free base form at physiological pH. This reaction, of first order (random spontaneous molecular dissociations analogous to radioactive decay), is sufficiently slow for its rate to be easily measured at room temperature. The rate is unaffected by the presence of any (scavenging) nucleophiles capable of reacting with the aziridinium ion that is formed. Generally, the kinetics of this first step in the alkylation reaction do not influence the outcome of selectivity studies using the NBP reagent [29], which are usually done at elevated temperatures.

These characteristics of the *first* step of alkylation by DMC are also characteristic of the first step of classical SN1 alkylations, and from quite early times a few toxicologists have mistakenly assumed that the mechanism is indeed SN1 [31]. It was difficult to reconcile this assumption with observations that DMC and the other 2-chloroethylamines are selective for "soft" nucleophiles such as the ring nitrogens of DNA (SN2 mechanism). As already discussed, the relevant feature of SN1 reactions that accounts for the lack of nucleophilic selectivity is the extreme reactivity of the carbonium ion (reaction 2, Figure 16). In fact, the aziridinium ion (DMA) formed from DMC (Figure 20) is not a carbonium ion. It reacts only very slowly with water and other neutral oxygen species. This was established during the 1930s and 1940s using simple but ingenious titrimetric methods, and confirmed in 1965 by NMR spectroscopy [32]. We have determined DMA by liquid chromatography using an acidified aqueous mobile phase [16]; the injection solutions

were stable for at least 38 h. Note, however, that if the solution is made alkaline, aziridinium ions react rapidly with the hydroxyl anion (Figure 20). I emphasise the lack of hydrolytic reactivity of DMA to recall that when such aziridinium ions react with nucleophiles, they do so selectively, via an SN2 mechanism, as shown in Figure 20 for alkylation of N-7 of a guanosine base.

Scavenging of nitrogen mustards While the initial first-order reaction of DMC to DMA is irrelevant to the mechanism of the actual alkylation reaction, it is clear that, unlike "ordinary" SN2 reagents, DMC will not be scavenged on its way to the nucleus by endogenous nucleophiles (Section A.2.3). The aziridinium ion DMA will be scavenged, because it an SN2 reagent that has a significant lifetime in water. The situation is, as it were, somewhere in between those of classical SN1 and (single step) SN2 reagents.

As already discussed, N-selective alkylation of DNA bases is mutagenic, but not exceptionally so unless the alkyl group has particular properties. DMC is officially carcinogenic, but it is much less potent than the bifunctional analogue mustine, which causes crosslinking within or between strands of DNA, as shown in Figure 21. These crosslinking reactions are sequential and temporally independent. Crosslinking is a likely explanation for the damaging effects of mustine and analogues, because normal DNA repair mechanisms would be defeated. Doubts have been expressed, however, because it was thought that at least one of the functional groups of mustine should react with endogenous nucleophiles before the molecule has had time do diffuse to the nucleus, and direct measurement of DNA crosslinking is difficult. Quite likely, the fraction of an administered dose that reaches the nucleus intact may have been underestimated, because the intact 2-chloroethylamino function is unreactive towards scavenging nucleophiles; in this sense, and in this sense only, the action of mustine is similar to that of SN1-like anticancer agents such a methylnitrosourea.

Appendix A.3: Conclusion

The causal relation between ionising radiation and cancer and other genetically-mediated effects is reasonably well understood both qualitatively and quantitatively. Although genotoxic chemicals act by similar mechanisms, quantitative aspects, particularly dose-response relationships, remain pooly understood. This situation is due in part to the complexity of the subject, but also to some remarkably inappropriate choices of investigative methods, and to a failure to understand the consequences of the different chemial reaction mechanisms.

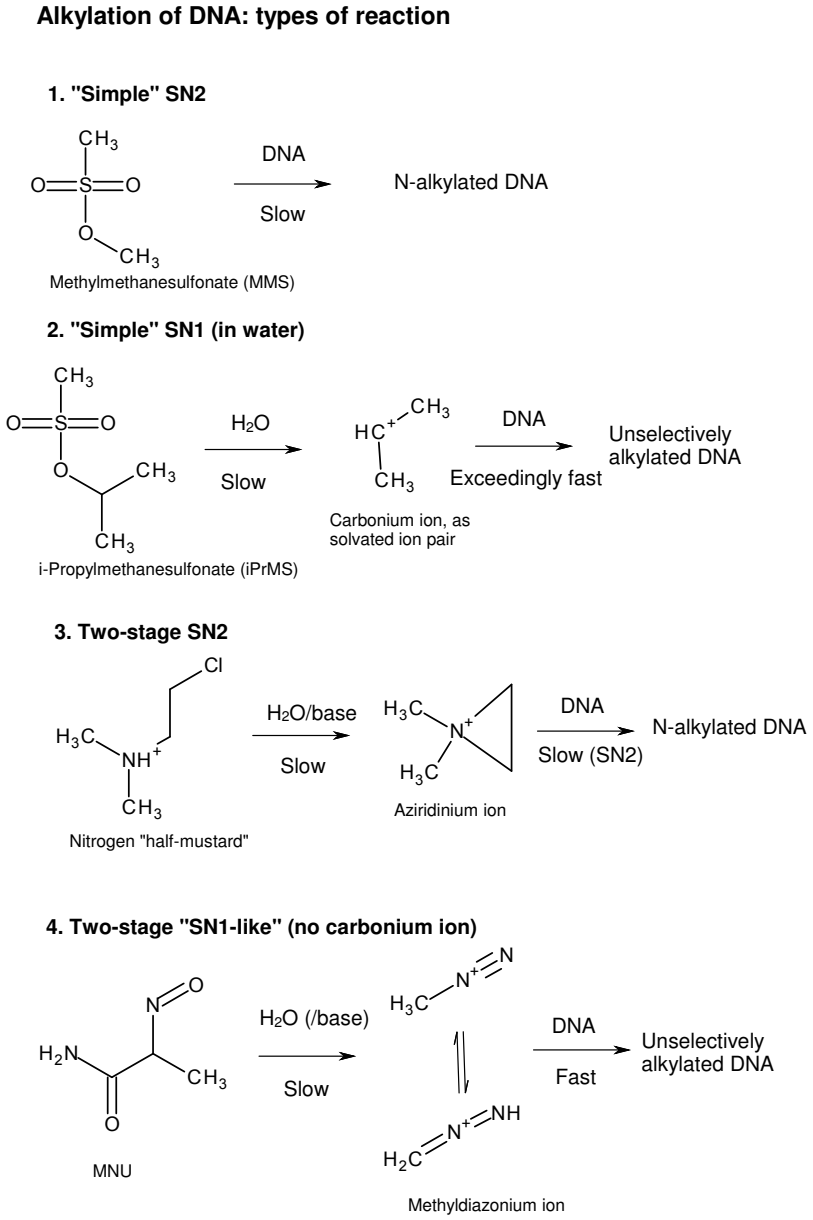

Figure 16: Reaction mechanisms of commonly studied alkylating agents

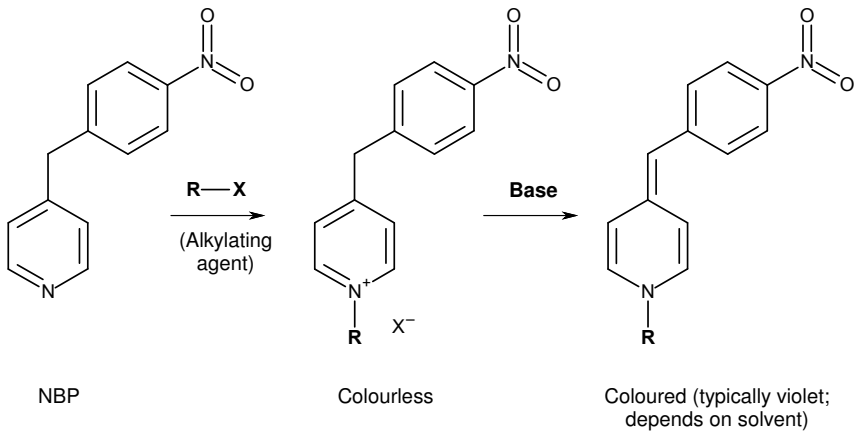

Figure 17: Reaction of NBP reagent with an alkylating agent.

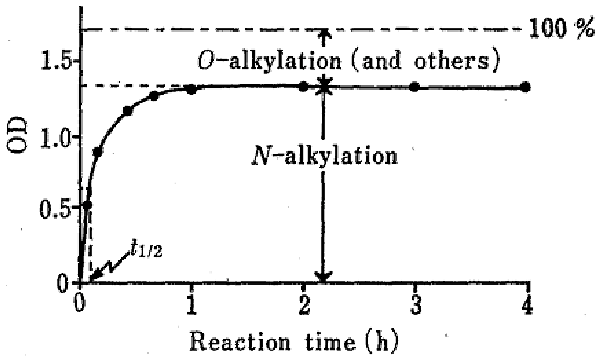

Fig. 1. Time Course Changes in the OD of the Benzene Extract from the Reaction of Benzyl Tosylate according to the Standard Analytical Method described in Experimental

Figure 18: Measurement of reactivity of an alkylating agent with NBP reagent [22], and selectivity with respect to water and buffer components. OD = optical density (absorbance; proportional to concentration)

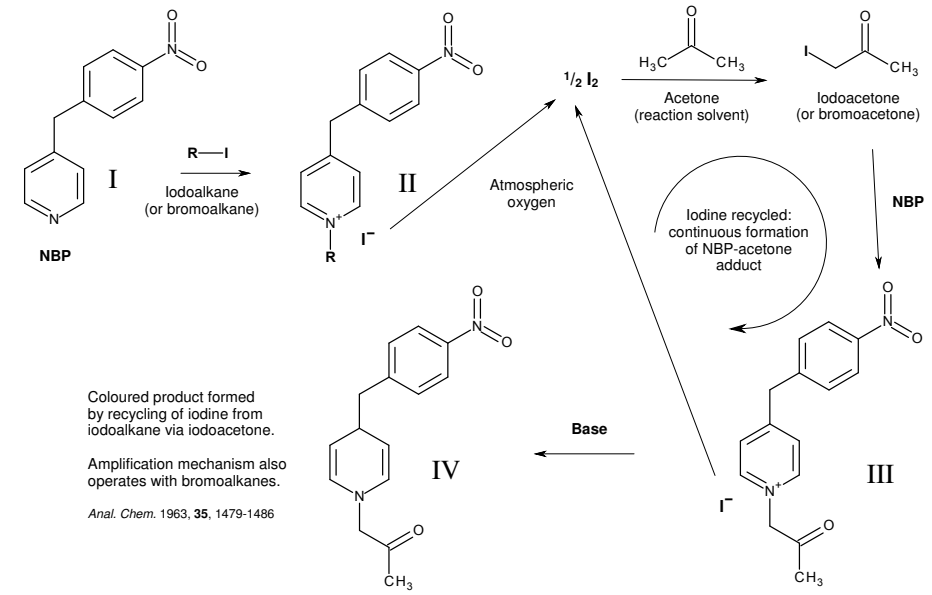

Figure 19: Oxidative recycling of iodide (or bromide) amplifies response to the corresponding haloalkanes

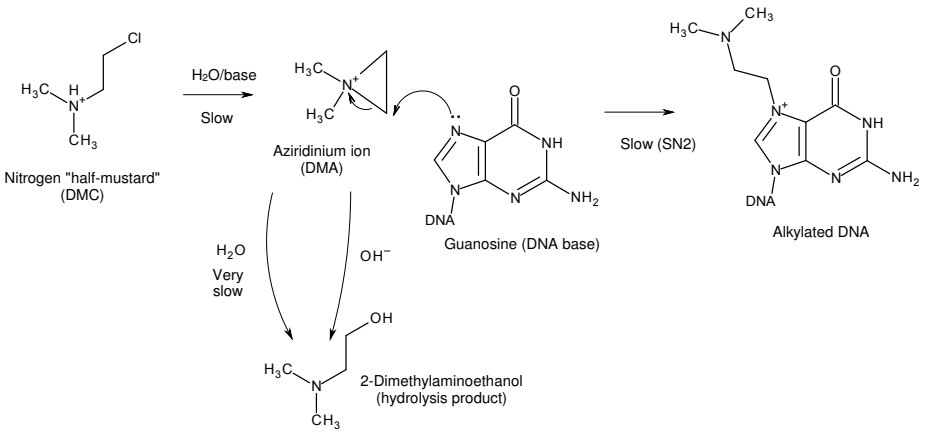

Figure 20: Reaction of a "half nitrogen mustard" with DNA and with water

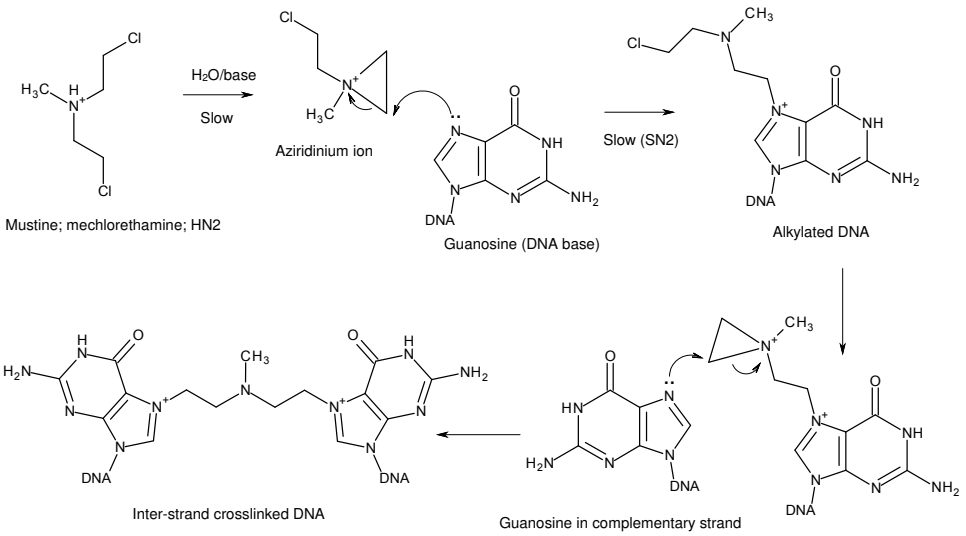

Figure 21: Reaction of mustine with DNA

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