Mechanistic Relationship among Mutagenicity, Skin Sensitization, and Skin Carcinogenicity

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Twenty organic *Salmonella* mutagens, seven of which (including benzo[*a*]pyrene) are established skin carcinogens, and one of which (2-chloroethanol) is a well-defined noncarcinogen to skin, have been evaluated for skin-sensitizing activity using the local lymph node assay. The relative mutagenicity of the agents to *Salmonella* was also established. Fourteen of the chemicals were positive in the local lymph node assay, including the seven skin carcinogens. 2-Chloroethanol was inactive as a sensitizing agent. We suggest that a variety of factors contributes to the lack of sensitizing activity of the remaining six bacterial mutagens: extremes of intrinsic chemical reactivity, high water solubility reducing dermal translocation, and inappropriate dermal metabolism. Two reference skin-sensitizing agents (an oxazolinone and fluorescein isothiocyanate) were established as *in vitro* clastogens after their recognition as nonmutagens to *Salmonella*. These data imply that mutagenicity, rather than simply activity in the *Salmonella* assay, is a primary stimulus for electrophilic sensitization and carcinogenic initiation in the skin. We conclude that genotoxicity data for an agent can provide indications of the agent's potential to induce skin sensitization and that genotoxins which are skin-sensitizing agents have an enhanced potential to initiate skin carcinogenesis. We suggest that common, albeit individually distinct, structure–activity relationships underpin genotoxicity, skin sensitization, and the initiation of skin carcinogenesis. These relationships should simplify the hazard evaluation of chemicals and contribute to a reduction in animal usage. Several predictions of skin carcinogenicity are made based on the data presented. **Key words:** lymph node assay, mutagenicity, skin carcinogenicity, skin sensitization. *Environ Health Perspect 101(1):62–67*

There are health consequences of both acute and chronic exposures of the skin to reactive chemicals. Dermal exposure to some chemicals may cause allergic sensitization such that subsequent skin contact will result in erythema and/or edema. Other chemicals may induce cancer at the site of their chronic, repeated application. Erythema and edema are temporally distinct and have not hitherto been related, as evidenced by their independent assessments when screening chemicals for toxicity. The chemical induction of skin cancer is triggered by dermal exposure to either natural alkylating agents (electrophiles) such as ß-propiolactone (see Table 1), hydrolytically derived alkylating agents such as *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (MNNG), or agents such as benzo[*a*]pyrene (BaP) and 7,12-dimethylbenz[*a*]anthracene (DMBA), which can be metabolized in the skin to an electrophilic epoxide derivative. Covalent binding of such electrophiles to nuclear DNA can lead to DNA mutations, and, under appropriate conditions, tumors. As expected, such agents are mutagenic in assays such as the *Salmonella* test. In contrast, although the induction of skin sensitization depends on interaction of the sensitizing (allergenic) chemical with epidermal Langerhans cells and the initiation of specific T-lymphocyte responses, the nature and site of this interaction in the skin is not precisely defined.

Skin sensitization and skin carcinogenicity almost converged in the early 1960s. In 1963, Old et al. (*1*) reported the ability of BaP, DMBA, and 3-methylcholanthrene to sensitize guinea pig skin, but not mouse skin. These authors suggested that the selective immunogenic activity was associated with the selective carcinogenicity of these agents to mouse skin but not to guinea pig skin. In particular, Old et al. asserted that chemically modified proteins triggered an immunological response in the guinea pig that prevented carcinogenicity.

This possible role of protein interactions in carcinogenicity rapidly faded when Brookes and Lawley (*2*) reported a correlation between the extent of binding of polycyclic aromatic hydrocarbons such as BaP to mouse skin DNA and their respective carcinogenic potencies to the skin. The sensitizing activity of BaP in the guinea pig was confirmed in 1978 (*3*), but in 1987 Klemme et al. (*4*) demonstrated sensitizing activity for both BaP and DMBA in the mouse, without making reference to the earlier negative results of Old et al. In 1967, Stevens (*5*) reported the results of a study in which the ability of 44 organic chemicals to sensitize guinea pig skin was discussed. Three of the sensitizing chemicals listed by Stevens, *N*-methyl-*N*-nitrosourea (MNU) (*6*), MNNG (*7*), and ß-propiolactone (*7*), have since been reported as skin carcinogens, but no attempt to correlate skin carcinogenicity and skin sensitization had been reported. Likewise, more recently, Roberts and Basketter (*8*) ex-plored the structure–activity relationship between the physicochemical properties of six long-chain sulfonic esters and their sensitizing properties to mouse skin, but there was no discussion of the possible skin carcinogenicity of these agents.

A major limitation of the earlier skin sensitization literature is the subjective nature of the observations: edema and erythema were scored on a scale from light pink to bright pink (*5*). However, the recently developed local lymph node assay (*9,10*) has placed measurement of sensitization on an objective footing (see below), and this led us to reevaluate the possible relationship between the electrophilicity/mutagenicity of chemicals and their skin-sensitizing properties and skin carcinogenicity. The mutagenicity of a chemical might provide an indication of its skin-sensitizing potential, and this, in turn, might provide a rapid and convenient indication of its carcinogenic potential to the skin—the definition of which is both time and resource consuming.

Methods

All chemicals and reagents are commercially available, were of the highest available purity, and were used as received (*10*). The biological techniques we used are well established. We determined skin-sensitizing activity by exposing the dorsum of both ears of groups of four CBA/Ca mice to the test chemical in olive oil/acetone (4:1) on 3 successive days. Five days after the initiation of exposure, we injected mice with tritiated thymidine (^{3}HTdR; 30 µCi/animal), and 5 hr later we measured lymphocyte proliferation in the pooled auricular lymph nodes local to the treated ears in terms of incorporated radioactivity.

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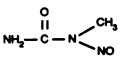
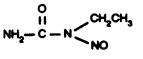
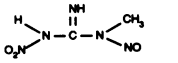
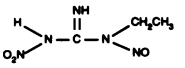
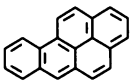
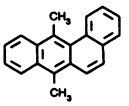
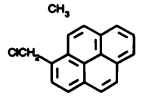
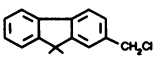
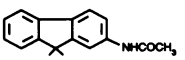
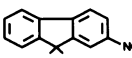
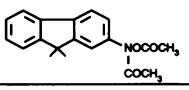
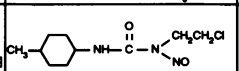
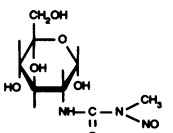
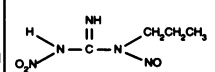
Table 1. Activity of 20 mutagens as skin-sensitizing agents in the local lymph node assay[a]

Abbr. name [CAS No]	Chemical Structure	Incorporation of ^{3}HTdR into local lymph node (test/control ratio) following exposure to the % solution (w/v) shown.								
		0.025	0.05	0.1	0.25	0.5	1.0	2.5	5	10
MNU [684-93-5]			2.7	7.1	15.4					
ENU [759-73-9]			0.4	0.7	1.0		2.7			22.3
MNNG [70-25-7]			27.5	60.4	78.3					
ENNG [4245-77-6]			5.7	9.6	8.4		15.3			
β-PRO [57-57-8]		1.5					13.0	19.9		
BP [50-32-8]						17.6	19.2	27.0		
DMBA [57-97-6]		7.6				17.7	15.6	17.7*		
1-CMP [1066-00-6]		11.6	15.4	18.6						
2-CMF [91679-67-3]				5.7		10.8	18.3	22.1		
2CE [107-07-3]	$ClCH_2CH_2OH$							1.2**	1.0	1.6
2-AAF [53-96-3]							1.6	1.9		
2-NF [607-57-8]							2.2	2.8		
2-AAAF [6098-44-8]							11.7	17.3		
MeCCNU [13909-09-6]						0.9	1.5	0.5		
STREP [18883-66-4]			0.6	0.9	0.9	0.5+	0.6+	0.6+		
PNNG [13010-07-6]			1.0	3.0	3.8		8.5			
MMS [66-27-3]	$CH_3SO_3CH_3$		1.0	0.6	0.7		0.7			3.6
EMS [62-50-0]	$CH_3SO_3CH_2CH_3$				0.6	0.8	1.0			0.6
DMS [77-78-1]					3.8	6.0	5.7			29.0
DES [64-67-5]							0.8	1.9		12.0

Abbreviations: MNU, methyl nitrosourea; ENU, ethyl nitrosourea; MNNG, *N*-methyl-*N*-nitroso-*N*′-nitroguanidine; ENNG, *N*-ethyl-*N*-nitroso-*N*′-nitroguanidine; ß-PRO, ß-propiolactone; BP, benzo[*a*]pyrene; DMBA, dimethylbenz[*a*]anthracene; 1-CMP, 1-chloromethylpyrene; ; 2-CMF, 2-chloromethylfluorene; CE, chloroethanol; 2-AAF, 2-acetylaminoflurorene; 2-NF, 2-nitrofluorene; 2-AAAF, 2-(*N*-acetyl-*N*-acetoxy)aminofluorene; MeCCNU, semustine; STREP, streptozotocin; PNNG, *N*-propyl-*N*-nitroso-*N*′-nitroguanidine; MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; DMS, dimethyl sulfonate; DES, diethyl sulfonate.

[a]Criterion for a positive response: test/control ratio ≥ 3. *Tested in DMF. **Also negative when tested as a 1.6% solution in 70% ethanol (test/control = 1.2). +Tested in ace-tone/water.

Activity in the local lymph node assay is expressed as the ratio of test to control incorporation of ^{3}HTdR (T/C) (*10*). In practice, chemicals which under these conditions elicit a 3-fold or greater increase in ^{3}HTdR incorporation relative to vehicle-treated controls are considered to have skin-sensitizing potential (*10*). We determined mutagenicity to *Salmonella typhimurium* in the absence of auxiliary metabolism using strain TA98 for the aromatic alkylating agents, strain TA1535 for the simple alkylating agents, and strain TA98 (+S9 mix) for BaP, DMBA, 2-acetylaminofluorene (2-AAF), and 2-nitrofluorene (2-NF) (*11*). In two instances the clastogenicity of established sensitizing agents was measured in cultured mammalian cells. The genotoxicity data for these two chemicals are referred to in the text but will be published elsewhere (Ashby, in preparation). We used a colorimetric assay for alkylating activity employing the colorless reagent *p*-nitrobenzylpyridine (NBP), which yields a deep-blue product upon alkylation of its pyridine nitrogen atom (*12*).

Results and Discussion

To initiate either carcinogenesis or skin sensitization, a biologically significant level of DNA or protein adduction, respectively, must be achieved in the appropriate epidermal target cells within the initial few hours of exposure. This suggests a possible overlap of the structure–activity relationships for mutagenesis, skin sensitization, and skin carcinogenesis. For these initially nonspecific interactions with protein and DNA in the skin to occur, three conditions must be met. First, the chemical, or a skin-derived metabolite, must be electrophilic and therefore capable of covalent reaction with nucleophilic sites on proteins and DNA. Second, a sufficient quantity of the agent must penetrate the lipophilic stratum corneum of the skin. The partition coefficient of the chemical will clearly influence penetration; agents with low coefficients (i.e., high water solubility) are relatively disadvantaged. Third, once in the epidermis, the agent must be sufficiently reactive to produce a biologically significant level of protein/DNA alkylation before it is dispersed from the site of application and/or metabolically detoxified. These physicochemical factors are subsumed in the relative alkylation index (RAI) suggested by Roberts and Basketter (*8*) for alkyl sulfonates.

Preliminary experiments established that the reference mouse skin carcinogens and bacterial mutagens MNU (*6*), *N*-ethyl-*N*-nitrosourea (ENU) (*13*), MNNG (*7*), *N*-ethyl-*N*-nitroso-*N*′-nitroguanidine (ENNG) (*14*), ß-propiolactone (*7*), BaP

(*7*), and DMBA gave a strong positive response in the lymph node assay (Table 1). The response observed for MNNG was the strongest yet recorded in this assay and was induced at dose levels similar to those used in the skin-painting cancer bioassay. The activity of ß-propiolactone depends on the electrophilicity of its strained lactone ring, which adds further structural diversity to this set of carcinogens. Similar strong sensitizing responses were seen for the potent mutagens 1-chloromethyl-pyrene (1-CMP) and 2-chloromethylfluorene (2-CMF), two aromatic chloromethyl compounds designed to model the ultimate carcinogenic electrophiles formed metabolically from polycyclic carcinogens such as BaP and DMBA (*15*). Comple-menting these strong sensitizing activities was the inactivity observed for 2-chloro-ethanol (2-CE), a weak bacterial mutagen and probably the most well-defined mouse skin noncarcinogen yet described (*16*). The inactivity of 2-CE as a sensitizing agent was established under both the present standard conditions of the test and when applied under the conditions used in the skin-painting cancer bioassay (a 1.6% solution in 70% ethanol). These data therefore indicate that genotoxic carcinogens (DNA reactive/electrophilic) can also elicit immunogenic effects via their inevitable parallel reactions with proteins.

The one *Salmonella* mutagen (2-CE) that was unable to express its intrinsic genotoxicity as carcinogenicity to mouse skin was also inactive as a sensitizing agent to mouse skin.

The skin-sensitizing and carcinogenic activities of BaP and DMBA depend on their metabolism in the skin to electrophilic epoxides, and it therefore became of interest to study the sensitizing activities of the two rodent liver carcinogens 2-AAF and 2-NF, which are known to require complex gut/hepatic/biliary metabolism to electrophiles by enzymes, some of which are not adequately represented in the skin. The inactivity of 2-AAF and 2-NF as sensitizing agents at the application concentrations studied was in contrast to the strong sensitizing activity observed for the direct-acting (S9 independent) mutagen 2-(*N*-acetyl-*N*-acetoxy)aminofluorene (2-AAAF), a model of the ultimate electrophile formed metabolically from both 2-AAF and 2-NF (Table 1). These data establish that some genotoxins are prevented from eliciting biological responses in the skin due to a failure of appropriate metabolism. It is possible that weak sensitizing activity would be observed for 2-AAF and 2-NF at elevated dose levels, but the intention here was to conduct comparative studies. The similar sensitizing activity of the two 2-fluorenyl derivatives, 2-AAAF and 2-CMF (Table 1), is probably related to the similar and bulky fluorenyl adducts they produce on proteins. From these data, we conclude that probably all genotoxic (DNA reactive) carcinogens have intrinsic skin-sensitizing potential, but this potential is only realized when the carcinogens are appropriately absorbed across the stratum corneum and, if necessary, when their metabolic conversion to an electrophile is achieved in the skin. These data also indicate that 2-AAAF, 2-CMF, and 1-CMP have carcinogenic potential to the skin.

The 13 aliphatic alkylating agents shown in the middle panel of Figure 1 display mutagenicity to *Salmonella* over a 10^7 dose range. Although each of these agents was already known to be mutagenic, they have not hitherto been tested concomitantly, and consequently the wide differences in their mutagenic potencies and minimum effective dose levels have not been known. It is probable that this dispersion of active dose ranges is influenced both by the different intrinsic reactivities of the agents and by the extent of formation and repair of O^6-alkyl guanidine adducts on DNA. The dose–response envelopes shown in Figure 1 are for mutagenicity; the shading indicates that those agents also act as skin-sensitizing agents (see also Table 1).

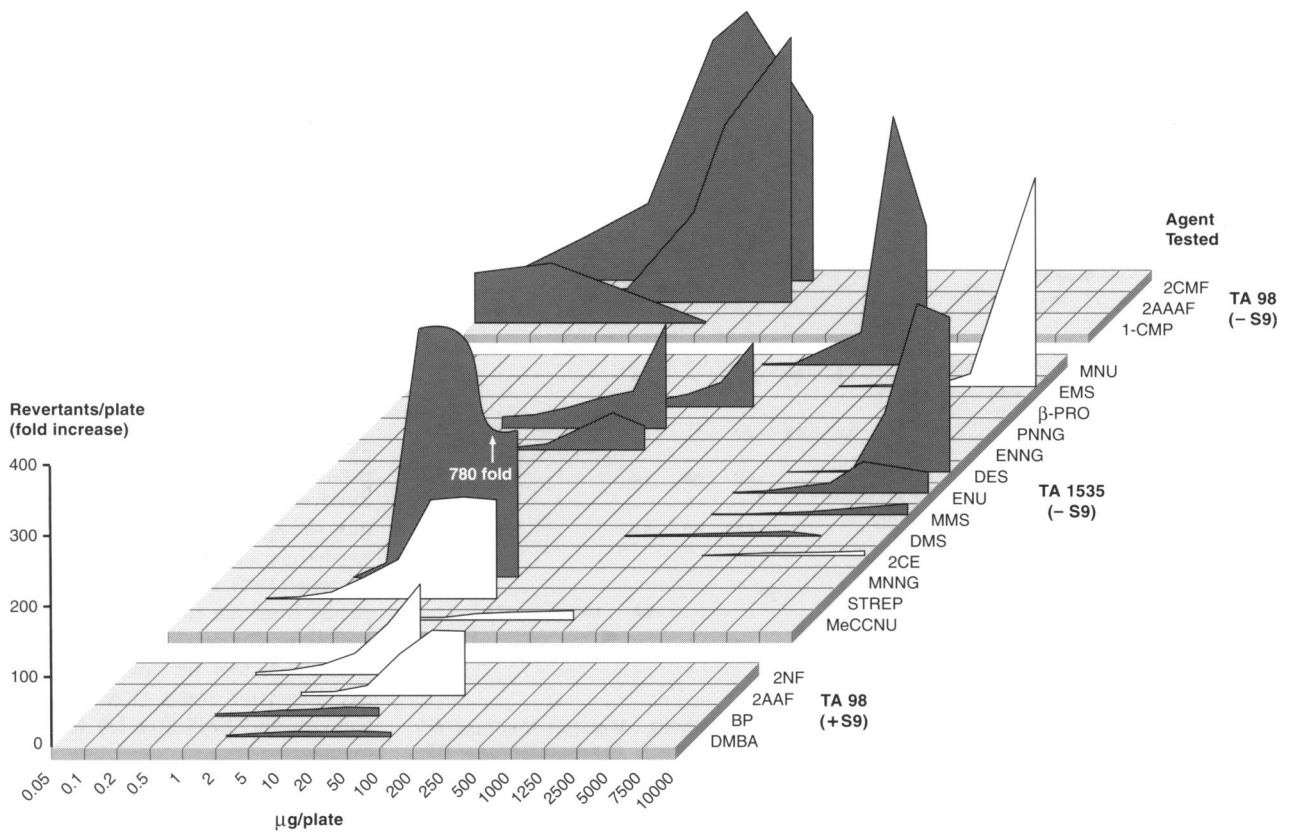


Figure 1. Relative mutagencity to *Salmonella* of 20 agents tested for skin sensitization. The mutagenicity dose–response envelopes are shaded for skin-sensitizing agents (see Table 1).

Given the preponderance of immunogenic chemicals shown in Figure 1, mutagens that were inactive as sensitizing agents obviously become of interest. The human antitumor agent semustine (MeCCNU) is active in slowing tumor growth by virtue of its ability to cross-link DNA, a property dependent on its hydrolysis to the bifunctional alkylating agent $ClCH_2CH_2N^+$. By cross-linking DNA, cell replication is impeded (*17*). The most likely explanation for the inactivity of MeCCNU as a sensitizing agent is therefore that it inhibits the necessary proliferation (*9*) of T-lymphocytes in draining lymph nodes. Consistent with this, application of MeCCNU 1 hr after exposure to MNNG led to a 75% attenuation of the concomitantly observed local lymph node assay response to MNNG (Table 2).

The failure of streptozotocin to elicit a sensitizing response is initially surprising because this nitrosourea is a rodent carcinogen and mouse somatic-cell mutagen when administered by intraperitoneal injection (*18*). The critical structural difference between streptozotocin and MNU is the sugar substituent on the former. The measured log P value for streptozotocin was -2.0 ± 0.3 (shake flask technique) compared to MNU's value of approximately 0, and this 100-fold enhancement of relative water solubility probably reduces the bioavailability of streptozotocin by attenuating its translocation across the lipophilic stratum corneum. In fact, streptozotocin was not completely soluble in the standard acetone/olive oil vehicle; consequently, at the highest three dose levels shown in Table 1, acetone/water (4:1) was used as vehicle, but even in this vehicle complete solution was not achieved at the highest dose level. All of the other agents in this study have log P values in the range 0–6, so a critical log P value of around -2 probably provides one key parameter for determining skin toxicities. It is interesting to note that exposure by intraperitoneal injection is not impeded by the protective lipid barrier of skin, thus explaining the genotoxic activities of streptozotocin in mice exposed by the parenteral route. [For a review of the rodent genotoxicity of streptozotocin, see Liegibel et al. (*18*)].

Dimethylsulfate (DMS), diethylsulfate (DES), and the two sulfonate esters [methyl methanesulfonate (MMS) and ethyl methanesulphonate (EMS)] are interesting because they demonstrate a wide range of reactivities as alkylating agents. Ehrenberg et al. (*19*) have measured the hydrolytic half-lives of three of these compounds and found DMS to have a $t_{1/2}$ of 0.1 hr and EMS and MMS to have $t_{1/2}$ values of 4 hr and 5 hr, respectively. These data suggest that the relatively high dose levels of DMS required to elicit mutagenicity to *Salmonella* may be due to its hydrolysis before reaction with DNA, while the similar high dose levels required for EMS and MMS may be due to their low intrinsic reactivity.

Likewise, although each of these alkylating agents gives a blue color in the NBP test, for DMS the color can be abolished by preincubation for 2 min with the nucleophile piperidine, while the other three compounds retain alkylating activity after 20 min of such preincubation (data not shown). It is relevant that the weak *Salmonella* mutagen 2-CE was of such low intrinsic alkylating activity that it required warming with the NBP reagent in order to develop a blue color, consistent with its noncarcinogenicity and nonsensitizing activity. The skin carcinogenicity of EMS and MMS is unknown, but the present data indicate EMS to be noncarcinogenic and MMS to have very low carcinogenicity to the skin. A limited skin-painting carcinogenicity bioassay of DMS revealed a negative response (*20*), but the present sensitization data indicate a carcinogenic potential that may be realized in an adequate bioassay such as that used by the National Toxicology Program for the evaluation of 2-CE (*16*). Diethysulfate and *N*-propyl-*N*-nitroso-*N'*-nitroguanidine are of unknown carcinogenicity to the skin, but based on the present sensitization data (Table l), we suggest that they have such potential.

Given this correlation between the skin carcinogenicity of a genotoxin and its activity as a skin-sensitizing agent, the role of genotoxicity data in the prediction of skin sensitization becomes of interest. When screening chemicals for genotoxicity, it is usual to conduct an assay for the induction of chromosomal aberrations (clastogenesis) on agents found to be nonmutagenic to *Salmonella*. The reason for this assay is that some chemicals are mammalian cell mutagens despite their inability to mutate prokaryotic DNA (*21,22*). This in turn raises the question of whether a chemical that is nonmutagenic to *Salmonella* but which is a mammalian-cell clastogen would have skin-sensitizing potential.

To address this possibility, we evaluated the genotoxicity of two established sensitizing agents active in the local lymph node assay (*23*) but whose chemical structures (*22*) indicated they were unlikely to be mutagenic to *Salmonella*: the oxazolinone 4-ethoxymethylene-2-phenyl-2-oxazolidin-5-one (EPO; I, Fig. 2) and the fluorochrome fluorescein isiothiocynate (FITC; II, Fig. 2). Both chemicals were reproducibly nonmutagenic to *Salmonella* but reproducibly clastogenic to cultured mammalian cells. The *Salmonella* assays were conducted using strains TA1535, 1537, 1538, 98, and 100 (*11*) in the presence and absence of induced rat-liver S9 mix to a high dose level of 1 mg/plate (FITC) or 5 mg/plate (EPO). The oxazolinone EPO was reproducibly clastogenic to cultured human lymphocytes (>50 μg/ml) when treated in the absence of S9 mix for 24 hr (Mackay and Fox, unpublished data). Fluoroscein isothiocyanate was reproducibly clastogenic to cultured Chinese hamster ovary cells (>400 μg/ml) when treated in the absence of S9 mix for 3 hr and harvested at 20 hr (Galloway and Armstrong, unpublished data).

Possible electrophilic sites within the structures of EPO and FITC that could be responsible for their clastogenicity and sensitizing activities are indicated in Figure 2, but why such reactive centers should fail to cause mutations in bacteria remains obscure. The concept that the Michael center in EPO and the NCS group in FITC are reactive exclusively to proteins is an initially appealing explanation for these selective mutagenic effects, but it remains speculative.

The above findings suggest that the mutagenicity of a chemical, as opposed to just its activity in the *Salmonella* assay, indicates carcinogenic and sensitizing potential of the chemical to the skin. Nonetheless, subtle differences in the structure–activity relationships for these three toxicities will exist. For example, all of the methylating agents studied (MNU, MNNG, MMS, and DMS) are more potent sensitizing agents (Table 1) than are

Table 2. Test/control (T/C) ratios of incorporation of ^{3}HTdR in mice receiving a single application of MNNG[a]

Test group	MNNG (% w/v)	MeCCNU (% w/v)	T/C ratio of ^{3}HTdR incorporation into local lymph node
1	0.25	Vehicle	15.3
2	Vehicle	1.0	0.7
3	0.25	1.0	4.3

Abbreviations: MNNG, *N*-methyl-*N*-nitroso-*N'*-nitroguanidine; MeCCNU, semustine.
[a]Mice (*n*=3) received a single application on the ears of either MNNG or vehicle alone, followed 1 hr later by a single application of MeCCNU or vehicle. Three days later T/C ratios were determined for pooled treatment groups.

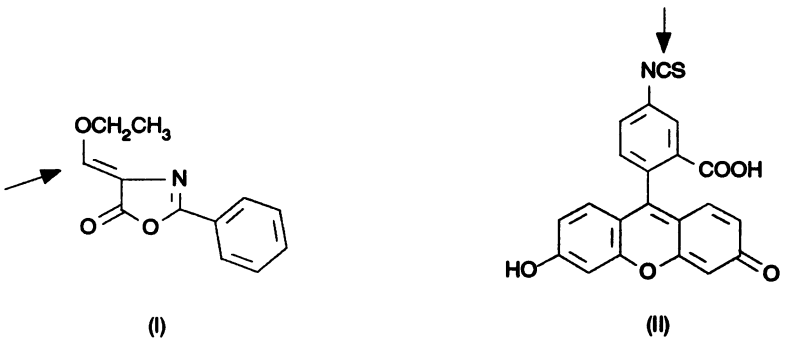


Figure 2. Chemical structure of 4-ethoxymethylene-2-phenyl-2-oxazolidin-5-one (I) and fluorescein isothiocyanate (II). The arrows indicate the site of possible electrophilicity on each molecule. Fluorescein isothiocyanate is sometimes drawn in the ring-closed spiro-lactone form.

their ethylating analogues (ENU, ENNG, EMS, and DES, respectively). This suggests that methylated proteins have higher immunogenicity, per se, compared to ethylated proteins. In contrast, although MNU and MNNG are more potent *Salmonella* mutagens than their respective ethyl analogues (ENU and ENNG), EMS and DES are clearly more potent mutagens than their methyl analogues MMS and DMS. These selective mutagenic effects are related to differences in the nature of the electrophilicity of the alkyl nitrosoureas/guanidines as compared to the alkyl sulfonates/sulfates. These chemical-class differences lead to different adduction profiles on DNA, which in turn can lead to different and organism-specific DNA-repair/mutagenic consequences (*24*). Similarly, differences in the mitogenic properties of a mutagen can lead to differences in the progression of initiated cells to carcinogenesis (*25*). Subtle differences are to be expected between the structure–activity relationships of mutagenesis, skin sensitization, and dermal carcinogenicity, despite an underlying coherence. This overall coherence is illustrated by the facts that the most potent skin-sensitizing agent described here (MNNG) is also the most potent of the present mutagens, and the weakest sensitizing agent (MMS) is also the weakest mutagen among those that are skin-sensitizing agents (Table 1; Fig. 1).

We therefore suggest that adequate evaluation of chemicals for genotoxicity (*21*) will also yield information on skin-sensitizing potential. In the case of metabolism-dependent genotoxins, the likelihood of the appropriate biotransformation occurring in the skin will have to be considered. Further, we suggest that the activity of a genotoxin as a skin-sensitizing agent provides strong indications of its potential skin carcinogenicity. It must be borne in mind, however, that relatively high dose levels are used when assessing the skin-sensitizing potential of a chemical because this may reflect the conditions of accidental human exposure (*10*). Chronic exposure of the skin to high, acute dose levels may not always be possible. Consequently, consideration of acute versus chronic allowable dose levels must accompany extrapolation of skin sensitization data to predict skin carcinogenic potential. Nevertheless, the dose levels used in this study were generally within the ranges used to establish the potency of the such skin carcinogens.

As it took more than a decade to qualify the initial observation that carcinogens are mutagens (*7*), it would be unwise to transmute the present correlations into inviolable relationships. Nonetheless, it is clear that a common structure–activity relationship underpins genotoxicity, skin sensitization, and skin carcinogenesis and that in the early stages of chemically induced skin carcinogenesis, adduct-related immunogenic as well as mutagenic activity is occurring. Such knowledge should aid and simplify the hazard assessment of chemicals.

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