Assessment of the Propensity for Covalent Binding of Electrophiles to Biological Substrates

by Robert M. Carlson*

Electrophilic character is associated with the ability of external agents to interact with centers of electron density in biological macromolecules and to cause the interruption or alternation of normal activity. With the observation of site specificity in mutagenic events, Pearson's hard/soft acid-based (HSAB) theory is presented as a useful concept in correlating chemical observations in the absence of detailed direct knowledge of the process. Methods for the evaluation of carbon electrophiles (e.g., carbocation character) as reactants are reviewed as potential physical parameters that could be applied in developing quantitative structure-activity relationships.

Background

The interaction of reactive organic chemicals with biological macromolecules often involves covalent binding (i.e., a substitution process such as alkylation, or conjugation) at nucleophilic (electron-rich) cellular sites susceptible to attack by an electrophilic (electron-deficient) component (1,2). In enzymatic processes this typically involves reactions with carboxylate, amino, hydroxyl, and sulfhydryl functionalities. Such functional group alterations would modify activity through the direct blocking of an active site or through conformational changes.

In genotoxic modes of action, modified DNA is generally considered to be unable to correctly express its design function and, if not repaired, may lead to a cytotoxic or a carcinogenic response (3). Moreover, it is not only apparent that the relative chemical reactivity of the alkylating agent is one of the dominating factors (1), but also that many other reactive electrophilic species, i.e., ultimate carcinogens (4–8), are derived from benign chemicals through normal metabolic processes.

For purposes of our discussion we will focus on DNA, which incorporates a variety of nucleophilic functionalities (e.g., carbonyls, hydroxyls, phosphate oxygens, amines, and heterocyclic nitrogens) where alkylations are well documented (9–13). Early studies examined the extent of total covalent binding (conjugation) and unsuccessful attempts were made to index this factor with carcinogenic potency (14). However, recent results suggest that a considerable difference exists in reactivity

among the various nucleophilic sites (15,16). In particular, alkylation at the O6 of guanine, O2, and O4 of thymine, and O2 of cytosine showed a correlation to a procarcinogenic event (17–22), while reactions at other positions resulted in enhanced levels of cytotoxicity.

The identification of actual (or potential) electrophilic sites in a chemical structure is the basis of a theory proposed by Miller (6). The strict application of this theory suggests that nonelectrophilic components of the chemical structure are chemically neutral and can be ignored. Ashby and Tennant (23) have subsequently presented a hypothetical chemical structure that incorporates functionalities typically associated with the designation of a chemical as a carcinogen (Fig. 1). Each of these functional units is electron-deficient.

Quantitative Assessment of Electrophilic Character

The evaluation of electrophilic character, as applied to chemical processes, can be approached through the principles of acid-base theory where electrophiles (acids) and nucleophiles (bases) can be classified as hard or soft, based on intrinsic characteristics of the center being considered. In this classification scheme, hard species typically have small atomic radii, a high effective nuclear charge, and are only slightly polarizable; soft species tend to be large and highly polarizable (Table 1). Pearson and Songstad (24) suggested the simple rule of thumb that hard electron-deficient centers prefer to bind with hard electron-rich centers and, likewise, soft electron-deficient species prefer soft electron-rich species. Klumpp (25) and Ho (26) have noted the power of the concept in correlating chemical facts in the absence

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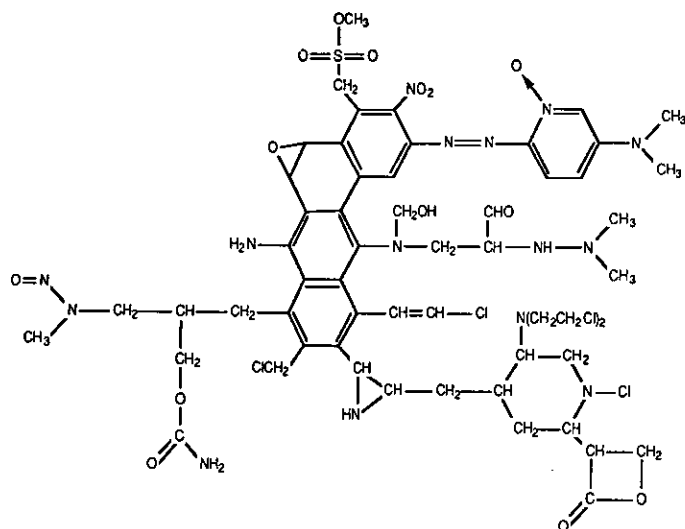
FIGURE 1. Hypothetical structure incorporating functional groups typically associated with chemical carcinogens. Adapted from Ashby and Tennant (*23*).

of detailed direct knowledge of the process under investigation.

If alkylation processes are examined using hard/soft acid base (HSAB) theory some important trends are observed for carbon acids (i.e., the electrophilic component): *a*) Carbon acids are classified as borderline hard/soft; *b*) hardness of carbon acids are in the order: $C_6H_5^+ > (CH_3)_3C^+ > (CH_3)_2CH^+ > CH_3CH_2^+ > CH_3^+$. This trend is ascribed to the more electropositive nature of hydrogen versus carbon; *c*) the more carbocation character that is observed during a reaction, the harder that center will be. This is particularly significant because carbocation content of a reaction is directly associated with first-order-processes (e.g., SN1) rather than second-order substitutions involving transition states involving charge dispersal (e.g., SN2 reactions); *d*) the hardness of the leaving group dominates in alkylations involving *n*-alkyl halides or tosylates (e.g., increasing hardness in the order RCl > RBr > RI); *e*) in cases where there are major hard-soft differences between the carbon center and the leaving group, the character of the alkyl component will dominate (e.g., $CH_3OCH_2Br \rightarrow$ hard).

The HSAB theory can be applied to DNA alkylation since the attack on the hard oxygen sites during methylation or ethylation can be correlated to carcinogenicity (*17-22*). Specifically, as the ratio of oxygen alkylation to that at other softer sites, such as the exocyclic amino group or the ring nitrogen increased, the carcinogenicity increased. Consistencies with HSAB are observed as ethylation results in more O-alkylation than methylation (*2*).

If a general application of the HSAB concept to site specificity relationships of carcinogenicity is to be effected, experimental validation methods must be applied. Several possible approaches are described below.

Physical Methods for Determining the Propensity of a Carbon Center to Sustain a Positive Charge

Various methods are available for the formation and study of carbocations in the gas phase (*27-29*). Some of these methods are outlined in Figure 2. Additional methods include chemiionization, penning ionization, fast atom bombardment, field ionization, and field desorption. Such techniques provide ions in an unsolvated environment. However, several of these methods are useful in common chromatographic detection systems, and if a standard reference compound is used, then could provide a relative index of carbocation stability.

Generating and Analyzing Carbocations in Solution

Special structural features [e.g., the trityl cation $(C_6H_5)_3C^+$ (*29,30*)] or extraordinary experimental conditions [super acids (*31*)] are required for the direct observations of carbocations. However, changes in the order of a kinetic process are indicative of mechanistic changes (e.g., SN2 → SN1). In principle, a continuum of SN1 percentages (e.g., carbocation content during the reaction) could be generated for correlation with biological data.

Table 1. Examples of hard/soft acids and bases (*24*).

Acids		Bases	
Hard	Soft	Hard	Soft
H^+, Li^+, K^+, Mg^{+2}, Al^{+3}, R-C = 0, C N	Hg^{+2}, Ag^+, Cd^{+2}, Cu^+, Pt^{+2}, CH_3^+	H_2O_2, HO^-, ROH, RO^-, F^-, Cl^-, SO_4, NO_3^-, PO_4^{3-}, CO_3^{-2}, CLO_4, NH_3, RNH_2, N_2H_4	R_2S, RS^-, I^-, SCN^-, $S_2O_3^{-2}$, Br^-, R_3P, CN^-, CO, RCN, C_2H_4, C_6H_6, H^-, R^-
Species with low electron affinity	Species with high electron affinity	Species with high electron affinity	Species with low electron affinity
Unoccupied orbitals are of high energy	Unoccupied orbitals are of low energy	Occupied orbitals are of low energy	Occupied orbitals are of high energy
Small ionic radius	Large ionic radius	Small ionic radius	Large ionic radius
Strong solvation	—	Strong solvation High charge	—

Thermal ionization $\qquad$ RX $\xrightarrow[\Delta\ \Delta]{}$ R$^+$+X

Photoionization spectroscopy (PES) $\qquad$ AB $\xrightarrow[h\upsilon]{}$ (AB)$^+$+e$^-$

$\searrow$ A$^+$+B

Electron ionization (EI) $\qquad$ AB $\xrightarrow[e]{}$ (AB)$^+$ $\longrightarrow$ A$^+$+B

Chemical ionization (CI) $\qquad$ R$^+$+RX $\longrightarrow$ RX+R
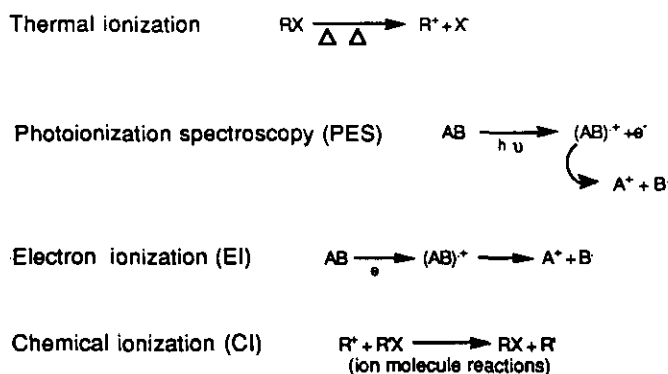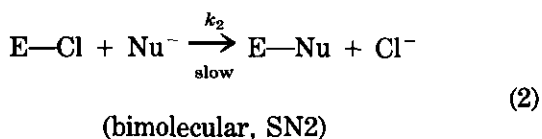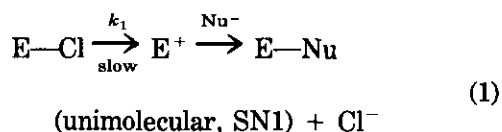(ion molecule reactions)

FIGURE 2. Examples of methods for studying carbocations in the gas phase.

Techniques are also available for the simultaneous determination of first and second order processes, and a correlation of first-order hydrolysis rate constants and partition coefficients to mutagenicity for a diverse series of compounds have been made in this laboratory (*32*). However, since only a small amount (e.g., 1%) of the hydrolysis was observed as first-order, the validity of correlating small changes in these low percentages was questioned (*33*). The logic of the kinetic approach and the evaluation of the method's limitations are described later.

Specifically, an electrophile within a polar medium in the presence of a nucleophile can rect via a unimolecular mechanism (1st-order process) or a bimolecular mechanism (2nd-order process). The two reaction pathways are shown below where k_1 and k_2 represent first- and second-order rate constants, respectively. E denotes the electrophile and Nu denotes the nucleophile.

$$\text{E—Cl} \xrightarrow[\text{slow}]{k_1} \text{E}^+ \xrightarrow{\text{Nu}^-} \text{E—Nu}$$

$$\text{(unimolecular, SN1)} + \text{Cl}^- \tag{1}$$

$$\text{E—Cl} + \text{Nu}^- \xrightarrow[\text{slow}]{k_2} \text{E—Nu} + \text{Cl}^-$$

$$\text{(bimolecular, SN2)} \tag{2}$$

Another description used for Eq. (1) is an SN1 process (substitution nucleophile unimolecular) and Eq. (2) is an SN2 process (substitution nucleophile bimolecular). Rate expressions for Eqs. (1) and (2) are shown below where Eqs. (3) and (4) correspond to (1) and (2), respectively.

$$\text{rate} = k_1 \text{ [E—Cl]} \tag{3}$$

$$\text{rate} = k_2 \text{ [E—Cl] [Nu:]} \tag{4}$$

The first- and second-order rate constants were determined simultaneously by assuming the overall rate is the sum of the SN1 and SN2 processes [Eq. (5)].

$$\text{rate} = k_1 \text{ [E—Cl]} + k_2 \text{ [E—Cl] [Nu:]} \tag{5}$$

Experimentally, the nucleophile concentration was set equal to the electrophile concentration, which explains the derivation of Eq. (6) from (5).

$$\text{rate} = k_1 \text{ [E—Cl]} + k_2 \text{ [E—Cl]}^2 \tag{6}$$

The integrated form of Eq. (6) is Eq. (7), shown below where $[\text{E}]_0$ represents the initial concentration of electrophile, $[\text{E—Cl}]_t$ equals the concentration of electrophile at time t, k_1 is the first order rate constant and k_2 denotes the second order rate constant

$$[\text{E—Cl}]_t = \frac{k_1[\text{E}]_0}{e^{k}1^t(k_1 + k_2[\text{E}]_0) - k_2[\text{E}]_0} \tag{7}$$

The experimental quantities of time and electrophile concentration were fit with BMDP statistical package (BMDP Statistical Software, Inc., 1440 Sepuveda Boulevard Los Angeles, CA 90025) that selects the best values for k_1 and k_2. The values of k_1 and k_2 for each electrophile indicate the relative contribution of SN1 and SN2 processes that result in the formation of E—Nu. A large k_1 value suggests the SN1 mechanism predominates and a large k_2 describes a system with a large SN2 component.

Three separate simulations were carried out. In the first simulation the effect of the initial values of k_1 and k_2 on their final values was determined. Initial values for k_1 and k_2 were found to be critical for the iteration to converge on the proper k_1 and k_2 parameters. If the initial k_1 and k_2 values are much large than the actual values (two orders of magnitude), the program will not fit the data to Eq. (8):

$$\text{C}_t = \frac{k_1\text{C}_0}{e^{k}1^t(k_1 + k_2\text{C}_0) - k_2} \tag{8}$$

Initial values of k_1 and k_2 that are smaller than the final values but greater than zero avoid this problem.

In the second simulation the program's ability to fit an equation that assumes first- and second-order rate data when given a large second-order rate constant was evaluated. Appropriate second-order rate data was synthesized with the following integrated second-order rate Eq. (9).

$$k_2 = \frac{[\text{P}]}{[\text{E}]_0[\text{E}]_t(t)} \tag{9}$$

where $[\text{E}]_t$ = concentration of electrophile at time t, $[\text{E}]_0$ = initial electrophile concentration, $[\text{P}]$ = product concentration at time t, and k_2 denotes the second-order rate constant. Time and concentration values were selected followed by fitting Eq. (8) to the data. By using initial values consistent with experimental data, final values of k_1 and k_2 were -0.019090/hr and 1.0653 L/mole hr, respectively. Similar values for k_1 and k_2 were obtained from experimental data of benzyl chloride derivatives and benzenesulfinic acid, where SN2 character dominates. Small negative values for k_1 indicate the data is second-order.
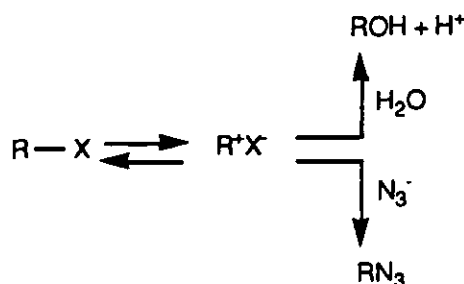
In the final simulation, data was synthesized using Eq. (8) with k_1, k_2, and C_0 at specified quantities. The

purpose of this simulation was to determine the magnitude of error in k_1 when k_1 is 0.1, 5, 10, 20 and 50% of k_2. Two sets of data were generated with three significant figures for each set of parameters. One set contained no experimental error, and the other had approximately 5% simulated experimental error introduced into the estimates of k_1 and k_2. The results of this simulation are shown in Table 2.

The results of these simulations raise serious doubts about the previously mentioned correlations observed in these laboratories. (Carlson, unpublished data). For example, in a typical experiment with 10% error in analysis, a 20% contribution of the first-order term would be required to recognize statistically meaningful differences among electrophile agents. However, as previously mentioned, we determined that for a wide variety of electrophiles only a small amount (1%) of observed hydrolysis is first order.

An alternative kinetic technique is the observation of selectivity versus reactivity in the presumed carbocation intermediates. Through the use of competition experiments, low selectivity among available nucleophiles would be observed with highly reactive carbocations. In the extreme this would represent an example of a diffusion-controlled process. In DNA alkylation, the sites of attack (O versus N) differ considerably in nucleophilic character and the observed O/N alkylation ratios should increase with a decrease in selectivity. The competition constant is defined in Eq. (10) *(31)* and is the ratio of the second-order rate constants with azide (k_n) and water (k_w). Low values for k indicate unselec-

tive (e.g., reactive) species. This technique allows for exploration of the character of carbocation species since the substrates that typically hydrolyze the fastest through an SN1 process are the ones that yield the most stable cations (e.g., low selectivity factors). The limitation in using this technique for comparison purposes is the necessity to assume that the same type of intermediates are, in each case, responsible for product formation.

$$\text{ROH} + \text{H}^+$$

$$\text{R} - \text{X} \rightleftharpoons \text{R}^+\text{X} \quad \begin{array}{c} \uparrow \text{H}_2\text{O} \\ \\ \downarrow \text{N}_3^- \end{array}$$

$$\text{RN}_3$$

$$K = \frac{k_N}{k_W} = \frac{(\text{yield of RN}_3) \cdot [\text{H}_2\text{O}]}{(\text{yield of ROH}) \cdot [\text{N}_3^-]} \quad (10)$$

Examination of Ambient Nucleophiles

The use of multiple electron-rich sites on the same molecule also provides an opportunity to examine the selectivity patterns of electrophilic attack. Two approaches emerge as convenient and appropriate. The first is the use of the extensive data already available on enolate alkylation. In this specific situation the choice is between alkylation at oxygen or carbon where the oxygen atom represents the harder of the two sites (Fig. 3). House notes specific examples of this phenomenon and refers to HSAB theory to explain the effect of leaving groups on increasing hardness (e.g., I < Br < Cl < O-SO$_2$R) and on the structure of the carbon component (e.g., more O-alkylation with secondary alkyl halides than with primary halides or with more polarizable allyl or benzyl halides).

An alternative protocol would be to expand on investigations where site-specific methylation of DNA base components (e.g., the free base, nucleoside, nucleotide) was examined. In these instances where there are many possible sites for alkylation, Singer and Grungerger *(16)* noted that "... no reaction with alkylating agents occurs *in vivo* that does not occur in *in vitro* model systems." Moreover, they have determined that "... within the limits of analytical systems available, alkylating agents reacted at the various sites in similar
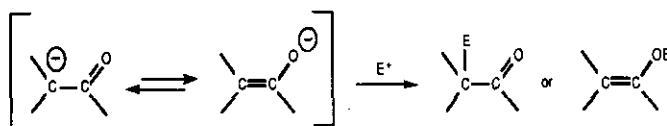
Table 2. Final simulated values of k_1 and k_2 derived from Eq. (8)[a], under a variety of initial input scenarios.

Input values		Final values			
		No error[b]		5% error[b]	
k_1[c]	k_2[c]	k_1	k_2	k_1	k_2
0.001	1.0	0.001232 (23%)[d]	0.988 (0.2%)	0.013 (1200%)	0.957 (4.3%)
$(k_1 = 0.1\% \ k_2)$					
0.1	2.0	0.12139 (1.4%)	1.9999 (0%)	0.058470 (41%)	2.165172 (8.2%)
$(k_1 = 5\% \ k_2)$					
0.1	1.0	0.099863 (0.14%)	0.999823 (0.02%)	0.157744 (58%)	0.830379 (17%)
$(k_1 = 10\% \ k_2)$					
0.2	1.0	0.200930 (0.47%)	0.996323 (0.37%)	0.165671 (17%)	1.099041 (10%)
$(k_1 = 20\% \ k_2)$					
0.5	1.0	0.502224 (0.44%)	0.993791 (0.62%)	0.555933 (11%)	0.817919 (18%)
$(k_1 = 50\% \ k_2)$					

[a] $C_t = \dfrac{k_1 C_0}{e^{k_1 t}(k_1 + k_2 C_0) - k_2}$; see text for an explanation of this equation. For these simulations C_0 was set at 1.0 mole/L.

[b] Simulations were run with either 0% "experimental error" (no error) in the initial input values for k_1 and k_2 or with 5% "experimental error" (5% error) introduced.

[c] Values of k_1 and k_2 are hr and L-mole/hr, respectively.

[d] Values in parentheses are percent errors associated with the final estimates of k_1 and k_2.

FIGURE 3. Enolate alkylation at either oxygen or carbon by an electrophile (E$^+$).

proportions *in vitro* and *in vivo*, except in those systems where there is rapid repair of O6-alkyl-G or 3-alkyl-A."

In earlier studies radiolabelled methylated products were used as standards. The ability to translate this technique to a broad range of potential electrophiles presents an obvious set of complicated synthetic and analytical tasks for each electrophile examined. In these laboratories (*33*) an analytical approach has been developed that depends upon three well-developed components: Attack on a specific site on the DNA base gives a characteristic UV spectrum that remains independent of the alkylating agent; the extinction coefficient of the product is also dependent only upon the characteristic chromophore present in the alkylated base. This allows for a quantitative assessment of the amount of each alkylated product that is present; and photodiode array ultraviolet (UV) instrumentation for obtaining a complete UV spectrum of a molecule during the course of a HPLC separation.

The techniques described have provided entrees to generate additional support for the site specificity of electrophilic agents in their interaction with DNA. This represents an approach to providing a reactivity parameter in quantitative structure-activity relationship based risk assessment.

The potential to utilize such isolated parameters has significant practical value, but care must be used in possible overstatement in processes such as mutagenesis or carcinogenesis. It is worth reminding ourselves that such processes are inherently complex and that we need to carefully heed the words of J. W. Drake (*34*):

> Thriving at the center of the bramble in which most of us have chosen to scratch out a living is a giant thornbush: inherent complexity. Most inquiries into genetic and chemical processes in biological systems are reductionist in nature and seek to determine how the organism or the cell carries out some process. We generally anticipate, with reasonable confidence based on historical results, that only one or at most a few mechanisms will turn out to be operating: the number of right ways to do something well is limited. The science of mutagenesis, on the other hand, encompasses the sum of all possible ways in which a highly evolved and extremely intricate process can go wrong. We can, therefore, anticipate that all possible routes to error actually occur with finite probabilities; conversely, we can also anticipate that our imaginations alone are unlikely to suggest each of these routes and that we will constantly be presented with delightful surprises.
>
> The problem, then, is not simply one of how mutagenesis occurs but, more realistically, is that of imagining all the ways in which it might occur, and then designing experimental attacks on these possibilities powerful enough not only to discredit the inappropriate answers but also to ferret out the as yet unimagined possibilities.

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