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Swain-Scott constants and alkylating agent drug design

Colin Paul Spears[1,2], Sang-Ihn Kang[2], Nitya G. Kundu[2,3], Tatyana Shamma[4], and George A. Olah[4]

[2]USC-Norris Comprehensive Cancer Center, University of Southern California Cancer Research Laboratory, 1303 North Mission Road, Los Angeles, CA 90033, USA. [3]Department of Chemistry, Indian Association for the Cultivation of Science, 700 032/2A & B Raja S.C. Mullick Road, Jadavpur, Calcutta-700 032, India. [4]Loker Hydrocarbon Institute, University of Southern California, University Park Campus, CA 90089, USA

ABSTRACT

Broad spectrum antitumor alkylating agents possess remarkably high Swain-Scott *s* constants, indicative of high discrimination for selectivity of reaction with strongly nucleophilic sites such as the N7 position of guanine. This review discusses relationships between *s* constants and intracellular product spreads, including the mutagenic products of O-alkylation; and structure-selectivity factors that determine *s* constants, such as reactivity, polarizability and softness of the alkyl substrate. We have achieved exaltation of *s* constants with selenone leaving groups, without the expected increased susceptibility to thiol-mediated cellular resistance, that contribute to the exciting promise of this new class of alkylating agents.

INTRODUCTION

Alkylating agents, particularly the oxazaphosphorines cyclophoshamide and ifosfamide, and the aromatic nitrogen mustards chlorambucil and melphalan, all ethylenimines, remain among the most widely used and well known anticancer agents. Dose-intensification strategies with bone marrow or peripheral stem cell rescue and cytokine support have contributed to increased clinical applications of ethylenimines in recent years. Protection of bladder urothelium with 2-mercaptoethanesulfonate has furthered the therapeutic ratios of the acrolein-producing oxazaphosphorines.

It is now apparent that selective protection of normal tissues may be obtained by pretreatment with amifostine (WR-2721, the radioprotectant), that can also diminish mutagenicities of electrophilic chemotherapies inlcuding the ethylenimines, platinating agents, and antitumor antibiotics (1,2). Thiol protection is an old idea (3-5), but its increasingly successful clinical application emphasizes the relative importance of the chemical kinetic behaviour of alkylating agents. A clinical trial of platinating DNA-adduct reversal by WR2721 has recently been initiated (6), inspired by a discovery that DNA cross-links in vitro may be reversed by thiourea (7).

Ethylenimines (ethyleneimines) are classical alkylating agents and electrophiles, and formally are described as *substrates* for nucleophilic substitution (8-19). They are primary, saturated alkyl halides whose typical behaviour in the product-forming step is bimolecular (nucleophile concentration-dependent) S_N 2 substitution, and thus they follow the normal nucleophilic reactivity order of primary alkyl halides (19-25). The ethylenimines share initial, usually rate-determining aziridinium ion formation (for each functional group, in sequence), as the explanation for their typically overall unimolecular kinetics (3,10,12-19,24,25). For the analogous sulfur mustards, internal bimolecular formation of a cyclic sulphonium salt is typically rate-limiting (21-28).

This kind of unimolecular rate-dependency on ring formation should not be confused with classical S_N 1 kinetics. True S_N 1 kinetics, with retention of

[1] Corresponding Author
e-mail: CPSpears@aol.com
Present Address: Dr. Colin Paul Spears, Huntington Cancer Center and Huntington Medical Research Institute 10 Congress Street, Pasadena, CA 91105 USA

configuration in the final and product-forming step, requires that the reactive intermediate species be a free carbonium ion of the type, $R_2NCH_2CH_2+$, suggested once for aromatic nitrogen mustards (24) but this would appear to be very unlikely (29-31). Even the weakest nucleophiles, such as solvent water, combine with primary carbon substrates with S_N 2, Walden inversion of tetrahydral carbon in the product-forming step. Solvolyses of even most secondary alkyl substrates also should usually be regarded as S_N 2 (30,31).

Second-order rate constants for nucleophilic substitution of aliphatic alkyl halide substrates in protic media, show the reactivity order: sulfur > nitrogen > oxygen nucleophiles, with ionized nucleophiles more reactive (an increase in Swain-Scott n value by about 1 unit for each negative charge). In solvolytic reactions with nucleophiles in protic media, the reactivity order of alkyl halides is $I^- > Br^- > Cl^- > F^-$ (20-26,28,32-36). This represents a complete reversal of the gas phase reactivity order (37,38), for example, for Menshutkin product formation by methyl iodide and ammonia (39), indicative of the determining role of solvation energies in the aqueous biological reactions and in solvolysis of alkyl halides in general. Unlike Menshutkin product gain in ΔG, increased solvation-stabilization of products in corresponding solvolytic S_N 2 reactions are more associated with loss of ΔG and a decrease in barrier height in the transition state (37-40).

THE SWAIN-SCOTT EQUATION

Ehrenberg, Osterman-Golkar and colleagues (41-47) and others (48-59,61) have provided convincing evidence for the hypothesis of Loveless (62) that increased alkylation of weakly nucleophilic oxygen nucleophiles in DNA, by substrates with low selectivity, is associated with increased mutagenic and carcinogenic potency.

The selectivity of a saturated, primary carbon substrate for nucleophilic substitution can be expressed as its Swain-Scott s constant, from Eq.[1], the linear free energy relationship (LFER) (33) frequently used in the carcinogenesis literature for correlation with biological data (42-53):

$$\log (k_y/k_o) = s(n_y - n_o) \qquad [1]$$

in which k_y/k_o is the ratio of second-order rate constants for reaction of a given substrate with a given nucleophile, Y, and for hydrolysis, under the same reaction conditions. By definition, $n_o = 0.00$ for water, and $s = 1.00$ for the reference substrate, CH_3Br (20-24). Values of n_y are highly correlated with the logarithm of Ogston's F constant ("competition factor")(26) for aqueous substitution reactions of the ethylenesulfonium ion: $\log F = 0.929\, n_{CH3Br} + 0.126$ ($r^2 = 0.991$ for 11 nucleophiles [20], with $F \equiv 0.00$ for H_2O instead of the reported value of 1.72 (24) since the latter is essentially the uncorrected logarithm of 55.5 M water).

High s values (0.9 - 1.3), and bifunctionality, characterize the great majority of therapeutic alkylating agents (20,24,25,42). High nucleophilic selectivity has also been associated with a "lasting ability in water" in the case of epoxides and arene oxides (61), that confers an ability to reach intracellular targets. This presumes a normally reciprocal reactivity-selectivity relationship (31,63-65), a basis for Lawley's classification of low and high s constants as a spectrum of S_N 1 vs. S_N 2 reactivities (58-59). Compounds with low nucleophilic selectivity, i.e., low Swain-Scott s, generally tend to be more reactive, show relatively greater formation of O^6-guanyl DNA adducts, and possess greater mutagenic and carcinogenic potency, with maximal effectiveness at s values below 0.7 (42,43,48-52). Highly mutagenic and carcinogenic halomethyl ethers have reactive intermediates partially stabilized by alkyloxonium species (e.g., $CH_3\text{-}O^+\!=\!CH_2$) (63), which do not follow the "normal" reactivity order (66), and have greater sensitivity to basicity factors in the nucleophile than shown by alkyl halides However, chloroethyl epoxide does follow the classical order, with a relatively low s of 0.71 (48) by nucleophile product competition (20).

Correlations of s with mutagenicity or carcinogenicity was apparent in several early studies of the monofunctional alkylating agents, ethyl and methyl methane sulfonates and nitrosoureas (42,43). Not surprisingly, this relationship was much less apparent for bifunctional substrates (49,50), because of the potent biologic effects of crosslinking. However, s is related to a parameter of crosslinking, the clastogenic efficiency, given by the ratio of induced-X ring chromosomal loss to sex-linked lethal mutations, over a wide range of mono- and bifunctional substrates (50).

The presence of neighboring leaving groups (typically separated 3-10 Å) in crosslinking substrates, akin to the alpha effect in nucleophiles, contributes in various ways to reactivity (67), (as in Fig. 2), so that *s* and crosslinking functionality are often related parameters.

High selectivity for Swain-Scott, essentially anionic nucleophiles, is shared by therapeutic alkylating and platinating agents, and by peroxide oxygen (68-72), a major product of biological radiation (discussed below).

The very high *s* values of ethylenimines and platinating agents accounts for their high levels of substitution at runs of N^7-guanines in DNA, shown by Maxam-Gilbert sequence analysis (73-76). These regions have the highest electronegative potentials in DNA (77,78). Ionic effects of salt composition and concentration are important for influences on conformational and DNA-nucleophilic center availability (76), as well as on Swain-Scott reactivities of the alkyl substrate. For example, lithium chloride is slightly weaker than sodium chloride for competition with 4-(4'-nitrobenzyl)pyridine for mechlorethamine (C.P. Spears, unpublished). For *in vitro* living cells, the presence of zinc chloride confers significant protection against melphalan (79), which could occur by either effect. Interestingly, selenium and nicotinamide, both highly nucleophilic, stimulate recovery from alkylation damage (80,81).

INTRACELLULAR PRODUCT SPREAD BY THE SWAIN-SCOTT EQUATION

In principle, the product spread of a primary carbon alkylating agent in aqueous reaction with a mixture of nucleophiles is predictable, based on *s* of the substrates and *n* values of the nucleophiles, and assuming no interactions among the parallel, negligibly reversible reactions (20). That is, where Y is the nucleophile concentration, the sum of all products, $\sum P_x$, is given by Eq. [2],

$$\sum P_x = 10^{sn_1} \cdot Y_1 + 10^{sn_2} \cdot Y_2 + \ldots + 10^{sn_x} \cdot Yx \qquad [2]$$

Fig. 1 shows a theoretical product spread based on estimates of average *n* values and concentrations of intracellular nucleophiles for two different conditions: high reduced glutathione (GSH, 2 mM), panel A, and absent GSH, panel B. For the latter, the presence of B12s, the strongest nucleophile known (57), is postulated. The concentration of DNA is taken to be 15 mM, consistent with approximate figures of 5 pg DNA/cell, a cell volume of 1000 μ^3, and an average nucleotide M.W. of 325 daltons. The *n* values of O^6- and N^7-guanine are approximations (42-46,56). The concentrations are minimal values, because of nuclear, chromosomal, and mitochondrial concentration gradients. However, the predicted relative levels of O^6- and N^7-alkyl guanine adducts by this model are remarkably similar to published analytical data (51,53,55,57-59).

Loechler's analysis of Swain-Scott relationships points up the finding of relatively greater oxygen adduct formation by ethylnitrosourea, compared to diethyl sulfate, than expected (83). However, the alkyl diazonium ion, one of the intermediates of nitrosourea substrates, is unlike classical Swain-Scott substrates in having a significant susceptibility to basicity (the pK_a) in the nucleophile. For example Edwards' β is 0.191 for diazoacetone, vs. 0.074 for sulfur mustard, 0.000 for glycidol, a negative 0.052 for iodoacetate , and 0.006 for methyl bromide (32). The most important Edward equation (32,84), Eq. [3] is:

$$\log (K/K_o) = \alpha E_n + \beta H \qquad [3]$$

where (K/K_o) is the relative (to water) ratio of kinetic rates or of equilibrium data, and E_n is the polarizability of the nucleophile, represented by the electrode potential. Basicity (H) of the nucleophile is a function of pKa, and $\alpha + \beta$ are the constants for the substrate, representing relative susceptibility to polarizability and basicity, respectively. Edward's α values ranged from 1.68 for ethyl tosylate to 2.59 for iodoacetate (32).

There is a theoretically reciprocal relationship between the Brønsted and Swain-Scott equations (85), so that as basicity becomes more important, Swain-Scott nucleophilicity is less important. Competition for diazonium ion formation through homolytic vs. heterolytic pathways adds further kinetic complexity for that leaving group. In studies of methyl transfer by phenyl-dimethylsulfonium ions to Swain-Scott nucleophiles, both the Brønsted plots and Edwards' H constant show better correlations than *s* (86), since *s* is so predominantly a polarizability parameter (32).

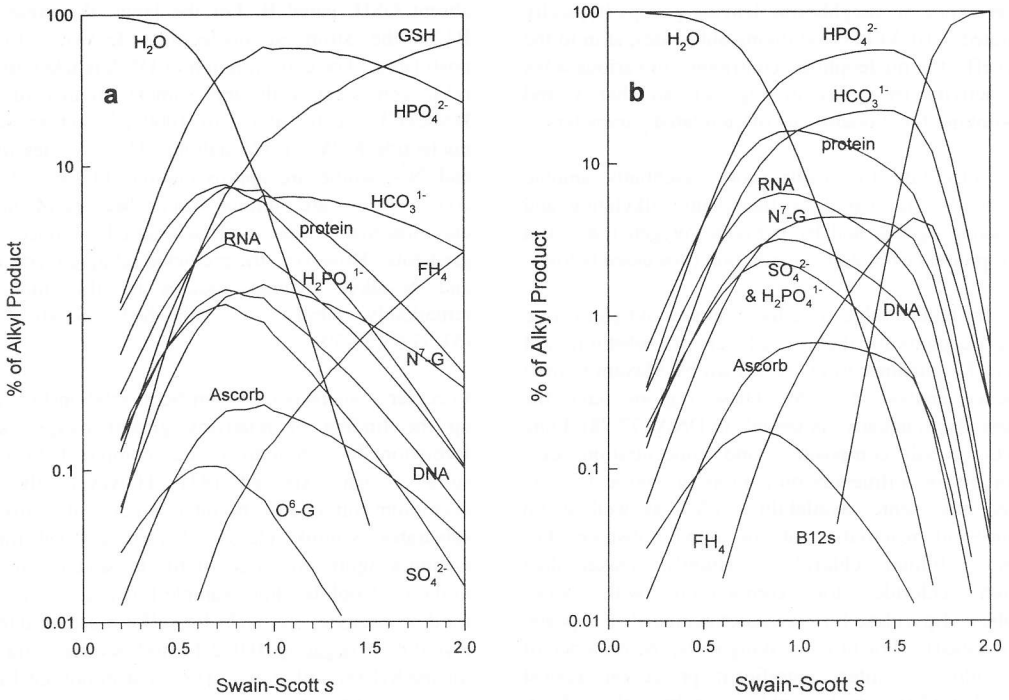

Fig. 1

Intracellular product spread vs. Swain-Scott *s* of a given alkyl substrate, by Eq. [2] (20); average nucleophile concentrations and their *n* constants (33,42,46): divalent phosphate, 76 mM, 3.8; reduced glutathione (GSH), 2 mM, 5.1; bicarbonate, 8 mM, 3.8; water, 33 M, 0.00; monvalent phoshate, 124 mM, 2.5; protein, 50 mM, 3.0; DNA, 15 mM, and RNA, 30 mM, 2.8; N^7-guanine in DNA, 4 mM, 3.5; O^6-guanine in DNA, 4mM, 2.0; ascorbate, 1 mM, 3.3; tetrahydrofolate (FH4), 0.005 mM, 5.4; sulfate, 20 mM, 2.5; and B12s, $2x10^{-10}$ mM, 10.4. Two conditions are presented, GSH present (left panel) or absent (right panel, with B12s present). In the absence of any strong ($n \geq 5$) nucleophiles, bicarbonate and FH4 would compete even more effectively with nucleic acid targets for alkyl product formation.

The Fig. 1 model is useful to demonstrate the phenomenon that when a strong nucleophile is present in substantial concentration, such as reduced glutathione, the peaks for weaker nucleophiles such as O^6-guanine show narrowing and leftward shift over the range of *s* constants. Thus, whether a given substrate will maximally alkylate a weakly nucleophilic, promutagenic site in DNA depends not only on *s*, but on the mix of nucleophiles present. It is interesting that in mutagenicity studies in barley, maximal mutations were obtained with alkyl substrates with *s* about 0.6 (42), similar to the O^6-G peak of Fig. 1a. Also, below *s* values of 0.4, it is apparent that competing hydrolysis becomes so dominant, that for poorly selective substrates, one may assume that other, non-Swain-Scott factors, especially Brønsted basicity and steric factors become important for prediction of the nucleophilic reactivity order.

The model could be modified to incorporate effects of secondary alkylating centers and their selectivities. For example, 1,2-dihaloethanes are well known to be

converted to episulfonium ion substrates through initial alkylation of thiols. Formation of alkyl phosphotriesters, and esterification of carboxyl groups, in many cases will be expected to lead to at least low levels of alkylating activity by the majority of these new, secondary centers, that typically would be expected to have both low reactivities and low selectivities to Swain-Scott (anionic) nucleophiles, but would probably have relatively higher sensitivities to Brønsted basicity in the next nucleophile, than the original substrate.

It is immediately apparent that an *s* constant greater than 0.8 is essential, if less than 50 % hydrolysis of the alkyl substrate is to be obtained. Another conclusion is that no single nucleophilic site, $n<6$ or so, can be stoichiometrically alkylated by a classical substrate. On the other hand, nucleophiles in low concentrations such as B12s with an estimated *n* of about 10 (82) conceptually could be alkylated by over 90 percent by extremely high *s* substrates such as cisplatinating agents. Adding complexity may be consideration of alterated reactivity order by hydrophobic microenvironments of membranes and in enzyme active sites, and concentrations of cations, as noted.

High *s* antitumor alkylating and platinating agents react principally at N^7-guanine sites in DNA, especially at runs, a result of high α in the substrates, with high sensitivity to the most electronegative sites. However, possession of high *s* confers susceptibility to thiol-mediated mechanisms of drug resistance. The product spread model of Fig. 1 predicts that thiol protection can provide about one log of protection, by realistically achievable levels of intracellular concentrations, and in fact, drug resistance against classical alkylating agents typically is rather modest, just 2- to 10-fold. However, the 'pseudo-first order" semilog cell-kill kinetics of electrophilic antitumor agents (alkylating and platinating agents and many antibiotics) is the basis for major clinical benefit by dose-intensification, such that just a several-fold increase in dose is associated with geometric increases in percentage cell kill.

Nitrosoureas have increased alkylation of the weakly nucleophilic but rather basic O^6-guanine, a highly toxic event. Repair of O^6-alkyl guanine adducts by O^6-alkylguanine transferase confers resistance to these agents, with thiol mechanisms being less important in general. The intermediate *s* value of 0.90 of *bis* (2-chloroethylnitrosourea)(20), $CH_3CH_2N(N=O)CONHCH_2CH_2Cl$, (BCNU) that is halfway between values for monofunctional nitrosoureas and ethylenimines, is evidence of aziridinium ion formation by the alkyl halide carbamoyl portion of the molecule, and may explain why thiol mechanisms of resistance are more often observed than for simple nitrosoureas.

DETERMINANTS OF NUCLEOPHILIC SELECTIVITIES OF SUBSTRATES

Although *s* and *n* values formally refer to rate data, and this was the intent of Swain and Scott (33), they in fact represent thermodynamic parameters, as discussed by Edwards (32), including ion-complex and solubility constants. The colinearity (20) of Swain-Scott n_{CH3Br} (33) with Ogston's *F* for sulfur mustard (24-26), determined by titration of pH change and chloride ion release, makes the same point.

Bartoli and Todescu (87,88) effectively developed Edwards'-type energy relationships for prediction of reaction rates based on basicity and solvent parameters, and on polarizability, with useful correlations for both aromatic and aliphatic nucleophilic substitution. In particular, their success is based on use of nearest atom bond refraction data (89), required for polyatomic nucleofugic groups.

Consideration of the nearest neighbor, bond refraction approach in 3-membered ring systems is of interest. Bond refractions from Table 5 of Le Fèvre (89) are for carbon-carbon, 1.286, for carbon-oxygen, 1.53 (ethers), for carbon-nitrogen, 1.55, and for carbon-sulfur, 4.57. Swain-Scott selectivities of ethylene phenonium ions include *s* constants of 0.5-0.6 for chloride and 0.22 for tosylate (90,91); of ethylene oxides, *s* of 0.6-0.86 (48,50); of ethylenimonium ions 1.1-1.3 (20); and ethylene sulfonium ion, 0.95-1.15 (20,33). Thus, the relationship does not hold for sulfonium ions, possibly related to a potential loss of selectivity with increased reactivitites. Little difference in anionic vs. basic Swain-Scott nucleophiles is apparent for any of the 3-membered ring systems, i.e., these appear to be fully linear with the reactions of methyl bromide (except for hydroxide ion).

Methyl perchlorate (34,35) and methyl 4-nitrobenzene-

sulfonate (92) both have greater susceptibility to basic than anionic (and heavy atom) nucleophiles compared to methyl bromide. In the case of 4-nitrobenzenesulfonate, separate linear correlations exist between Swain-Scott *n* and rates for substitution by primary, secondary, and tertiary amines (originally developed by Hall for epoxides and chloroacetate, see discussion in [92]), with subsets among these.

In a recent analogous case, methyl transfer by phenyl-dimethylsulfonium ions to Swain-Scott nucleophiles, Brønsted plots and Edwards' H constant also showed better correlation than *s* (86). Hydrolysis of α-glucopyranosyl fluoride, by C-F bond cleavage, has an *s* of 0.18 among Swain-Scott nucleophiles; with a pyridinium base as leaving group, $s = 0.03$ (93).

Differences in nucleophilicities among large series of nitrogen nucleophiles have been well characterized by Ritchie's N_+ , which is a combined solvent/nucleophile parameter for prediction of rates of cation/anion combination (93-95). The overall success of this approach again underscores the central role of solvent; the cations typically are carbenium ions, however, such as from esters (a recent example is retinyl acetate [96]), secondary and tertiary structures, and aryl diazonium ions, that tend to have less susceptibility to polarizability factors than saturated, tetrahydral-intermediate Swain-Scott substrates.

Quantitative approaches to the hard and soft acids and bases (HSAB) concept that appear to be less specific than Edwards' equation (84) and that of Bartoli and Todescu (87), have also been proposed (88). There are relationships between the basicity/polarizability parameters of Edwards, in Klopman's general treatment of chemical reactivity, in which the softness of a saturated carbon substrate acid may be equated with its tendency to accept electrons in chemical bonds *in solution* (97) , and for which such frontier-controlled reactions are favored by very polar, high ε solvents (88).

In the very broad review of nucleophilic reactivities of Edwards and Pearson (68) , a conclusion was that "high polarizability results from the existence of low-lying excited states which, when mixed with the ground state, produce polarity." Another was that "certain highly reactive nucleophiles are characterized by having empty orbitals available which are relatively low in energy." In chemical physics, polarizability is the dipole moment induced by an electric field of unit intensity (p.41, Ref. 89).

Table II of (32) gives the comparative reactivities of Swain-Scott nucleophiles for saturated carbon, Platinum (II), and peroxide oxygen, reactivity orders that are well correlated with each other over a 6-log range of second-order rate constants. The similarities in the cancer therapeutic armamentarium, of ionizing radiation with peroxide oxygen generation, the 'radiomimetic' (98) ethyleneimines, and Platinum(II), surely must relate to this. The similarity of trivalent nitrogen, such as with hydroxylamine-O-sulfonate, in its nucleophilic reactivity order (68,99) is interesting for drug development.

A central role of polarizability in *s* of saturated electrophilic carbon is apparent in the strikingly linear relationship of *s* to refractive index, shown in Fig. 2 for 23 substrates, mostly simple alkyl halides, all classic Swain-Scott substrates. Notable is that β-halo substituents cause exaltation of polarizability reflected in increased refractive index and *s* value, beyond that of methyl iodide which some investigators had considered an upper limiting value (96). Many bifunctional alkylating agents thus may have increased *s*, like our Fig. 2 dihaloalkanes, which is rather like the alpha effect of nucleophilic reactivity, as discussed by Bartoli and Todescu (87) and others (63,67,68).

Nicolescu-Duvaz and colleagues in studies of substituent effects in aromatic nitrogen mustards concluded that an increase in antitumor therapeutic effectiveness was correlated with the presence of somewhat bulky ortho substitution (102-105). It seems possible that such ortho moeities could be within close enough to the charged, reactive ethylenimonium ion intermediates to increase polarizability of the reactive electrophilic centers through London forces. (Protonation decreases polarizability of carbon [89], as an aside). London forces, also referred to as dispersion or induced dipole-induced dipole interactions, vary with $1/\text{radius}^6$ between two atomic centers (106,107). Hence, the dramatic effects of proximity of polarizable atoms on nucleophilic selectivity (108,109), such as in Fig.2.

In his classic review, Bunnett (63) considered the

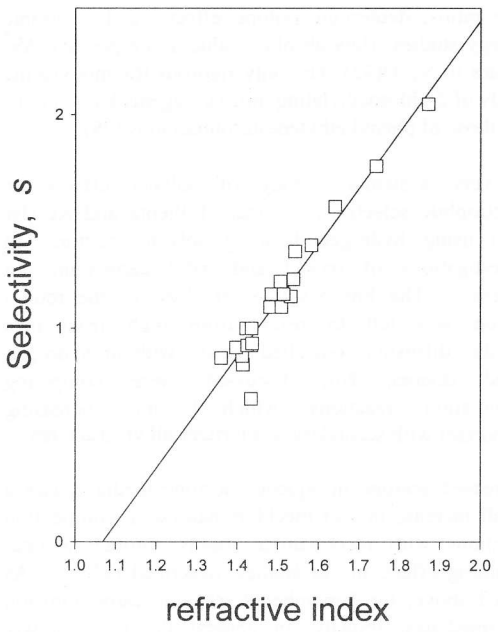

Fig. 2

Swain-Scott *s* vs. refractive indices (sodium D-line, [101]) for twenty-three alkyl halides and other simple saturated carbon substrates with acidic leaving groups, *s* constants from Refs. 20-23, 33, and 102 (normalized to methyl iodide *s* = 1.23), and C.P. Spears, unpublished (by NBP competition). Values of *s* and n_D by substrate: ICH_2CH_2I, 2.05 and 1.871; ICH_2I, 1.76 and 1.7425; $BrCH_2I$, 1.57 and 1.641; $ClCH_2I$, 1.39 and 1.5822; melphalan, 1.39 and 1.596; $BrCH_2CH_2Br$, 1.36 and 1.5420; CH_3I, 1.23 and 1.538; n-$CH_3CH_2CH_2I$,1.22 and 1.5058; CH_3CH_2I, 1.16 and 1.5133; $ClCH_2Br$, 1.16 and 1.4838; $S(CH_2CH_2Cl)$, 1.15 and 1.5313; ICH_2COOH, 1.10 and 1.5079[13]; $BrCH_2COOH$, 1.10 and 1.4804[50]; $ClCH_2COOH$, 1.00 and 1.4351[55]; CH_3Br, 1.00 and 1.4218; epichlorohdyrin, 0.93 and 1.4361; n-$CH_3CH_2CH_2Br$, 0.92 and 1.4343; CH_3CH_2Br, 0.92 and 1.4239; $(CH_3O)_3PO$, 0.91 and 1.3967; aziridine, 0.87 and 1.4120; oxirane, 0.86 and 1.3597; methyl methanesulfonate, 0.83 and 1.4138; and ethyl methanesulfonate, 0.67 and1.4335.

operation of London forces, among at least 17 factors contributing to nucleophilic substitution, as a major influence. For example, Bunnett et al showed marked increases in selectivities of benzyl chloride analogues by the presence of o,o'-substituents in reactions with anionic nucleophiles (110-112). The *s* value of o,o'-diiodo-chlorambucil, by thiosulfate/NBP competition, log (k_y/k_0) = 2.46, is actually somewhat lower than that of parent chlorambucil (C.P. Spears, unpublished). Very high *s* constants have been reported for beta-carbonyl-substituted methanesulfonates, carbamoyl methylmethanesulfonate (1.4) and 2-oxopropyl methanesulfonate (2 ± 0.2) (42). This result could occur by the normal reactivity-selectivity principle and should be investigated further, particularly as it may relate to BCNU and other carbamoylate substrates.

A miscellaney of factors not discussed thus far should be mentioned. Addition of even small quantities of polar solvents to non-polar media causes marked acceleration of S_N2 reactions through solvent-assisted leaving group behaviour (88). Relatively few attempts have been made to place leaving group (nucleofuge) parameters into context (87), but Thornton's series clearly is a function of basicity (113). Exaltation of polarizability by π-conjugation results in increased reactivities, and decreased selectivities, such as by allylic halides (114-116). Little change in net polarizability may occur associated with saturation of phenyl rings (117). Alkyl chain length has an effect on nucleophilic selectivity. For example, ethyl methanesulfonate (*s* = 0.69) is less selective than methyl methanesulfonate (*s* = 0.89) (42,43) despite the slightly higher refractive index of the former (1.4335) over the latter (1.4138) (100). Selectivity decreases with increasing alkyl chain length for alkyl methane sulfonates, and with alkyl group branching near the leaving group (42). Of course, steric effects, as a result of secondary and tertiary alkyl structure, causing hindrance to S_N2-type nucleophiiic substitition markedly decreases such selectivity. For example, the NBP alkylating activity of *tert*-butyl chloride is 0.0013% of that of mechlorethamine (C.P. Spears and G.A. Olah, unpublished). Azide is a stronger nucleophile than thiophosphate in solvolyses of trityl chloride (118).

REACTIVITY-SELECTIVITY RELATIONSHIPS

Good correlations between *s* and NBP alkylating activities of primary, saturated carbon substrates have been observed using protic media (20,119). Rates of increase in NBP chromophore reflect competition for

concurrent hydrolysis. In plots of n vs.log (k/k_0) in NBP competition, the n = 0.00 intercept will give some insight into findings of greater or less than expected competing hydrolysis.

Simple primary alkyl substrates are expected to show normally decreased selectivity with increased reactivity (29,31,64,65). However, secondary alkyl substrates can show increased azide product formation ("incorporation") vs. solvolysis with increased reactivity (29,31). Secondary, tertiary, and caged alkyl halides show S_N1-type, rapid leaving group behaviour with formation of carbonium and carbenium ions. Rapid formation reflects stability of the carbonium ion, giving paradoxically positive reactivity-selectivity relationships (29,31).

The cyclization reactions of episulfonium and ethylenimonium ions are viewed as "internal S_N2" in nature (120), with displacement of halide ion by β-nitrogen or β-sulfur. Thus, reactivity-selectivity relationships among classical substrates for alkylation may be complex and run counter to expectations (64). Values of s for the aziridine, thiotepa, and for nor-nitrogen mustard, which are much less reactive than mechlorethamine, are actually lower (about 0.9) than their ethylenimine counterparts (about 1.1 - 1.3) (20). As noted above, protonation should decrease polarizability as well as increase reactivity (despite potentially greater desolvation energies), yet ethylenimines are more selective than aziridines by NBP competition. The gain in charge and reactivity by 2-chloroethylamines on formation of cyclic ethylenimonium ions might be expected by the Hammond postulate to make their transition states closer to "reactant-like," with decreased selectivity (30,31). We noted a slight increase in s of ethylenimines by aromatic carrier (20), which through inductive effects slows rates of ethylenimonium ion formation. Cation desolvation energies of ethylenimonium ions may be critical for determining their high selectivities. However, almost no published studies of differential kinetic solvent effects exist for this important class of agents (apart from octanol/water partition coefficients).

Methods for describing S_N 2 vs. S_N 1 behaviour (29-31) include Winstein m determination (low in S_N 1), rate-pressure relationships, added nucleophile rate-product correlations, criteria such as [k_{EtOH} /k_{HOac}] and OTs/Br rate ratios, deuterium isotope effects, and entropic energy studies (low absolute-value, more positive ΔS^* values in S_N 1)(42). The only rigorous thermodynamic study of 2-chloroethylating, mustard agents known to us are those of phenyl ethylenesulfonium ions (28).

A very systematic study of solvent effects on electrophile selectivity is that of Pienta and Kessler (97) using hydrogen bonding solvent mixtures in investigations of retinyl and trityl carbenium ion kinetics. The low Swain-Scott slope of the retinyl cation was felt to result from high reactivities at the diffusion controlled limit, without hydrogen bond donors. Not discussed were competing elimination reactions, which become increasing important with secondary and tertiary alkyl structures.

Increased acetone in aqueous acetone media causes a small increase in s of mechlorethamine in competition reactions with nicotinamide, partly through a rate-retarding effect, in our studies, described below. As noted above, for 3-membered ethylene carbonium ion intermediates, broadly, in general the more slowly reacting nitrogen mustards are slightly more selective or the same as the more reactive sulfur mustards, that are more selective than the extremely reactive selenium mustards (122,123), in keeping with normal overall reactivity-selectivity relationships.

NBP IN EXTRATHERMODYNAMIC STUDIES OF ALKYLATION

The reagent, 4-(p-nitrobenzyl)pyridine (NBP), has been long been known to be useful for a variety of kinetic investigations of alkylating agents. Alkyl-NBP products tend to show little change in extinction coefficient with structural variation in the alkyl moiety (by direct measurement of isolated products [120] or indirectly by Gilette plot [48]). NBP possesses nucleophilicity essentially the same as N^7-guanine in nucleic acids, n = 3.5 in aqueous acetone (20), 4-4.3 in water (45). Activation energies, enthalpy and entropy parameters have been determined with great precision for aryl-substituted episulfonium ions by use of the NBP reagent (28). Reaction rates and activation energies for NBP alkylation by aliphatic substrates show useful correlations with mutagenicity in *S. typhimurium* (TA100) (115-117). NBP assays have been adapted for

use with chemical systems (Fenton oxidation) intended to mimic microsomal enzyme activation (124). Some workers report problems with instability in the chromophore, especially with sodium hydroxide/ethyl acetate; this is not a significant factor using triethylamine alkalinization, however. We have found NBP a useful reagent for study of several LFERs, including Hammett σ and ρ constants, and Winstein m values, in addition to Swain-Scott *s* and *n* constants (20), in studies of alkyl selenium substrates (122,123,125).

The positive solvatochromism of alkyl-NBP chromophores, i.e., red shift or bathochromic shift toward longer absorption wavelength with increasing polarity (and protic nature)of the solvent, has been noted by Sawicki and Sawicki (126). This is interesting, because some substrates, such as melphalan, fail to show the red shift (C.P. Spears, unpublished). The likely explanation for positive solvatochromism is the greater stabilization of the dipolar excited state on transfer of the ion pair to more polar solvents, although destabilization of the ground state by polar solvents could also contribute (p.293 Ref. 88).

NICOTINAMIDE AS A PROBE OF SUBSTRATE SELECTIVITY

Nelis and Sinsheimer adapted the analytical assay for N-alkyl nicotinamide as a aqueous alternative to the NBP test (127). Advantages of nicotinamide (NA) as a trapping reagent for alkylating substrates include the excellent aqueous (and lipid) solubility characteristics and remarkable stability in the developed fluorochrome.

We have modified the NA assay (95), for product competition determinations of *s* and *n* constants, as described in Fig. 3. This shows the alkyl-NA fluorescence-inhibition curves for mechlorethamine by the presence of competing Swain-Scott nucleophiles over a several log concentration range. The overall pattern of curves is remarkably similar to our own results with NBP, for example, as carried out by Barbin et al (48) for chloroethylene oxide with a similar series of nucleophiles. As with the NBP method, *s* is calculated from the 10 - 90 % inhibition range of data, by Eqs. [4] and [5]:

$$\log (k_y/k_{na}) = \log (([Y]/[NA])([E_c/E_o]-1)) \qquad [4]$$

$$s = (\log (k_{y1}/k_{na}) - \log (k_{y2}/k_{na}))/(n_1 - n_2) \qquad [5]$$

Log (k_y/k_o) is the ratio of second-order rate constants for nucleophilic substitution by Y and NA, [NA] is constant, 50 mM, and (E_c/E_o) is the ratio of fluorescence of samples with competing Y present vs. NA alone (E_o).

Eq.[4] is used for calculation of *s* from results of just 2 different competing nucleophiles, e.g., thiosulfate anion and azide ion, pyridine, or chloride. For a series of nucleophiles, of course, *s* is simply the slope of log (k_y/k_{na}) values vs. established *n* constants.

An interesting promise of the NA assay is determination of *n* values for biologic molecules, macromolecules, and even intact cells, under physiologic conditions of temperature, pH, and viability, with little toxicity expected from NA (but with many biochemical effects, however, such as promotion of NADH-mediated DNA repair), and background fluorescences that are readily accommodated. Relative fluorescence may vary with the N-alkyl NA moeity, which can be checked by Gilette plot (48). As with the NBP assay (20), selectivity for N-alkylation increases with temperature (127), which can be related to the increased softness of excited states by temperature (18).

The Fig. 3 NA assay permits essentially full range study of mixed solvent effects on the behaviour of primary alkyl substrates, including the ethylenimines. An example of such a study is given in Fig. 4, that presents results of study of aqueous-acetone mixtures from 20% to 100% water. Not unexpectedly, increasing acetone decreased alkyl-NA product formation by mechlorethamine, perhaps through increased NA solvation by acetone (and by decreased rates of ethylenimonium ion formation). In contrast, $\log(k_y/k_{na})$ values increased for both thiosulfate and azide ion competition with increasing acetone, suggestive of increased reactivity of these nucleophiles, relative to pyridinyl nitrogen of NA, by their decreased aquation energies. Of interest, the net effect by taking both thiosulfate and azide results is a modest increase in *s* with increased acetone, from 0.91 in 100 % water to 1.025 in 80 % acetone.

SELENIUM AS A POLARIZABLE HETEROATOM

It seemed possible to us that placement of the very soft,

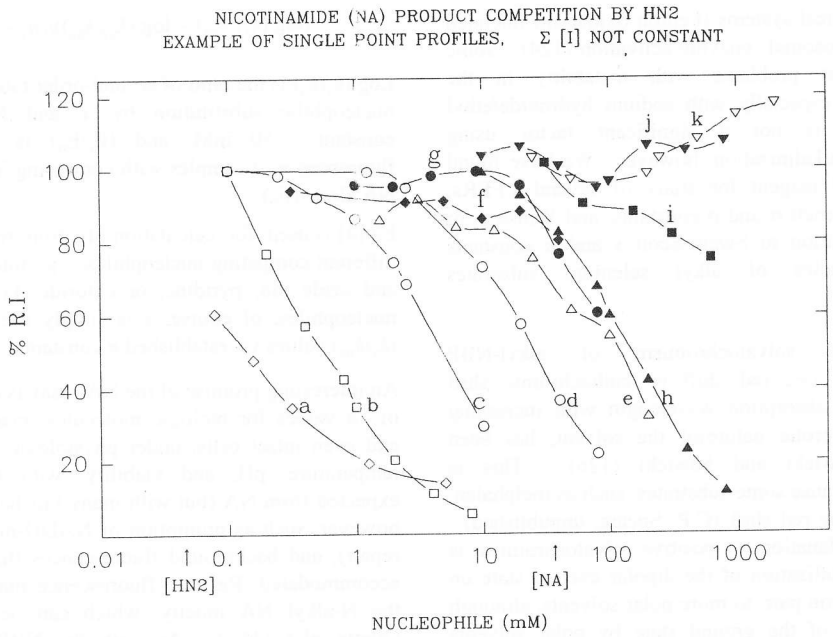

Fig. 3

Alkyl-nicotinamide (NA)product inhibition curves by added nucleophiles, vs. concentration of competing nucleophile, for mechlorethamine. At 4°, 60 µL of mechlorethamine HCl (0.5 mM freshly dissolved in 5 mM potassium phoshate buffer, pH 7.4), is added to 60 µL of premixed nucleophiles: 30 µL of NA, 250 mM in 5 mM potassium phoshate buffer, pH 7.4, plus 60 µL of Swain-Scott nucleophile in the same phoshate buffer, mixed,then incubated for 60 min at 37° (without shaking). The glass reaction tubes are cooled to 4° , and the fluorochromes of alkyl-NA are developed by addition of 15 % (v/v) acetophenone in 50 % ethanol:2M KOH for 15 min in the dark, before addition of 2.0 mL formic acid for ring-closure reaction at 90° for 10 min. Products are stable at 4°. Fluorescence readings at 23° are at 390 nm excitation and 430 nm emission. Blanks include absent mechlorethamine, NA, or absent competing nucleophile. Relative fluorescence readings, as a percentage of values without competing nucleophile (sodium salts for anions), are shown for (a) thiophosphate, (b) thiosulfate, (c) ethanethiol, (d)thiourea, (e) azide, (f) bromide,(g) nitrite, (h) pyridine, (i) acetate, (j) sulfate, and (k) nitrate. Note remarkable similarities to NBP competition data for chloroethylene oxide in aqueous acetone (48).

polarizable selenium atom near the carbonium reaction center would elevate *s* beyond what might be maximally achievable by ethylenimonium and episulfonium ions ($s \geq 1.3$). Selenium occurs naturally in a variety of oxidation states, including selenides (RSeR') and selenones (RSe(O)2R'). We reported the alkylating activities and cytotoxicities of a series of selenide alkylating agents, in which several LFER relationships were explored (122,123). The selenides showed relatively high sensitivity to solvent polarity for NBP alkylation (increased rates with increased aqueous component), compatible for overall rate-controlling,

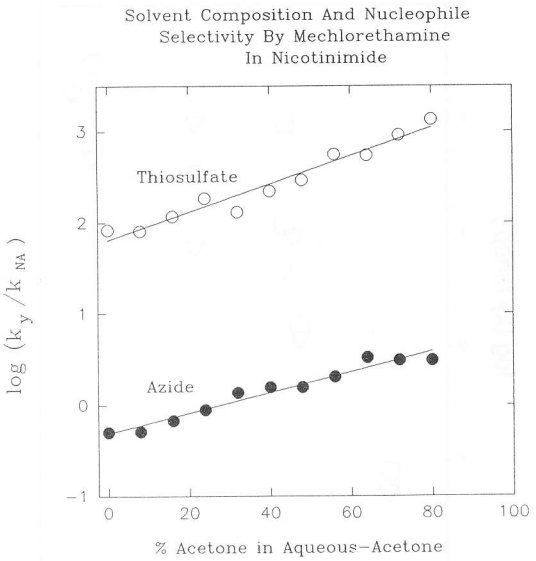

Fig. 4

Variation in *s* of mechlorethamine as a function of solvent composition. Values of log (k_y/k_{na}) for Y=thiosulfate or azide ion, vs. percentage composition acetone in the NA assay, performed as described in Fig. 3. Note increasing divergence of values with increasing acetone, so that based on either log (k_{S2O3}/k_{na}) or the difference between thiosulfate and azide values, *s* increases with acetone content.

S_N1 type, episelenonium ion formation (122). We also studied the effects of alkyl chain length and different leaving groups on selectivity, with some interesting clues for design of both *s* value and overall reactivity. such as with the propyl mesylate series (123), that achieved the highest *s* among the selenides, 0.87.

Increases in selectivity by the selenium atom in the selenide series were smaller than one might anticipate by the HSAB concept, and by the greater nucleophilicity of $C_6H_5Se^-$ (n = 7.6) than $C_6H_5S^-$ (n = 7.1) toward methyl iodide (calculated from methyl iodide n divided by 1.4, the *s* of methyl iodide in methanol [70]). The bond refraction (R_D in cm^3, sodium D-line) of Se-C is 6.0, compared to 4.6 for the C-S bond (89). For comparison, bond refractions for C-Cl, C-Br, and C-I are 6.5, 9.4, and 14.6 (63, 89).

We found however, decreased *s* and NBP alkylating activity by the diselenide, $ClCH_2CH_2SeSeCH_2CH_2Cl$, despite an R_D of 11.6 of the Se-Se bond and much decreased reactivity compared to the analogous monoselenide (122). A probable explanation is less participation by selenium, less ethylene selenonium ion formation, by the diselenide, in the activated transition state complex.

Oxidation of RSeR' selenides to $RSe(=O)_2R'$ selenones results in an remarkable change in mechanism of nucleophilic substitution, when R is an aryl moiety. $RSe(=O)_2$- becomes an excellent leaving group. This was noted by Reich who suggested the aryl selenones may be potent biological alkylating agents (128).

This is the case, in a series of 2-chloroethyl *para*-phenyl selenones for which we reported cytotoxicities and NBP alkylating kinetics (125). Compared to the corresponding selenides, the selenones possess broad lipid and protic solvent solubility characteristics that would predict rapid transport by passive diffusion through cellular membrames. Despite relatively slow rates of alkylation (NBP half-lives at 37° of 1 - 10 h), IC50s are submicromolar against L1210 leukemia and CCRF-CEM human lymphoblast cells, SKLU and SKMES human non-small cell lung cancer and several examples of other epithelial malignancies and CNS tumor cells (125).

An interesting aspect of structure-activity relationships among the *para*-aryl selenones is that electron-donating substituents (CH_3O-) are deactivating toward reactivity with nucleophiles but show enhanced cytotoxic potency. This is the opposite of the aromatic ethylenimines, which in general show both increased reactivities and cytotoxicities by substituents that increase basicity at their heteroatom reaction centers.

Increased nucleofuge basicity, for true leaving groups (in contrast to internal leaving group behaviour of sulfur and nitrogen in their respective mustards) is naturally expected to decrease reactivity. However, the initially rather nucleophilic selenone may be reactive through formation of an electrophilic pericylcic ethylene seleninic acid intermediate, that would also be expected to be less reactive as a result of *para*-aryl electron-donating substituents (130).

NBP-alkylating activities by the alkyl aryl selenones are

very high. As noted above, this reflects the ratio of second-order rate constants for alkylation of NBP vs. hydrolysis, $\log(k_{nbp}/k_o)$; however, relative contributions of the second, cross-linking 2-chloroethyl- electrophilic center are difficult to determine, for precise extraopolation to *s* value from alkylating activity alone.

The structure of ethyl phenyl selenone by X-ray diffraction techniques has provided evidence of the highly delocalized, polarizable nature of its electrons. Packing of the two ethyl group conformers in monoclinic crystals revealed almost no significant atomic interactions, with only Se–O···H–C interactions of phenyl hydrogens holding the crystal together (130).

Very high nucleophilic selectivities of aryl alkyl selenones are expected, as we have observed in $\log(k_y/k_{nbp})$ value determination by NBP competition. In Fig. 5 are shown results of 37° reactions for 16 h, of several oxygen, nitrogen, and sulfur nucleophiles in concentration-dependent competition with NBP, plotted against Swain-Scott n_{CH3Br} constants of these nucleophiles.

Selectivities of the selenones are extremely high, for preferential alkylation of nitrogen over oxygen nucleophiles. This is associated with a relatively greater spread between pyridinyl nitrogen and azide ion results than for *n* constants (3.6 and 4.0). For example, linear regression for $\log(k_y/k_{nbp})$ vs. *n* for *p*-methoxyphenyl 2-chloroethyl selenone for acetate ion, ascorbate, nitrite ion, pyridine, and azide ion gives Eq. 5, with $r^2 = 0.979$.

$$\log(k_y/k_{nbp}) = 1.46\, n_{CH3Br} - 5.30 \qquad [7]$$

That is, *s* of this selenone is 1.46 for nitrogen and oxygen nucleophiles, with the intercept value of -5.30 being an estimate of $\log(k_o/k_{nbp})$. Thus, alkylation of nitrogen nucleophiles is about 50,000- to 1,000,000-fold greater than hydrolysis, compared to the approximately 10,000 factor for ethylenimines.

It is unclear if reactivity for nitrogen nucleophiles is abnormally enhanced, or whether oxygen and sulfur reactivies are unexpectedly low, Fig. 5. Incipient formation of olefin-type alkyl reaction product could confer increased susceptibility to basicity in the nucleophile, but would not explain the greater $\log(k_y/k_{nbp})$ of azide, which is slightly less basic (pKa = 4.72) than pyridine (pKa = 5.23) (21-23,70) ('Abnormally high' azide reactivity has been seen with other carbon substrates, e.g., [83,97]). Alternatively, an electrophilic pericyclic seleninic intermediate may occur in an umpolung mechanism, in which the selenium atom competes with the electrophilic carbonium (or carbenium) ion for bond formation with sulfur.

In any case, the relatively enhanced nitrogen

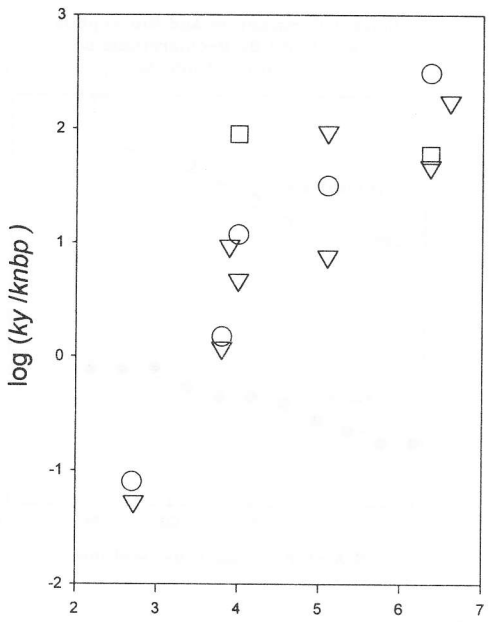

Fig. 5

Swain-Scott plots for organoselenium alkyl substrates, by NBP competition (20,122,123,125). Results are shown for 2-chloroethyl, *p*-methoxyphenylselenone (inverted triangles), bis(2-chloroethyl)selenide (circles), and 2-chloroethyl phenylselenone (squares). Nucleophiles and their n_{CH3Br} constants (15,16,25-27) were chloride ion (2.7), acetate (2.72), ascorbate (3.3), HPO_4^{2-} (3.8), bromide ion (3.89), azide ion (4.0), thiourea (4.1), iodide ion (5.04), ethanethiol (5.1), SO_3^{2-} (5.1), $S_2O_3^{2-}$ (6.36), and PSO_3^{3-} (6.6).

selectivities of the aryl alkyl selenones would predict the possibility of relatively less susceptibility to thiol mechanisms of intracellular drug resistance than for classical alkylating agents and platinating agent. And the low reactivities with oxygen nucleophiles would predict a lack of cellular resistance by increased O^6-alkyltransferase.

Both predictions are fulfilled, in cytotoxity studies of L1210/L-PAM and MER(+)-HT29 human colon carcinoma, with no evidence of cross-resistance by conventional resistance mechanisms of alkylating agents. In addition, no cross-resistance to epipodophyllotoxin-resistant human colon carcinoma is evident as well (131).

Additional studies will be needed to determine whether the highly promising IC50s in drug-resistant cell lines is fully relatable to the kinetics of nucleophilic substitution of the selenium substrates, or is a result of intrinsic selenium heteroatom metabolism. Given the well known anti-carcinogenesis effects of selenium, its important role in glutathione metabolism, the antitumor activity of analogues such as selenazafuran, and the requirement of some cell lines, such as human lung cancer, for selenium, it is surprising further work with this heteroatom as a pharmacophore has not been reported. Future investigations of the aryl alkyl selenones should include study of various strategies to increase overall unimolecular kinetic or metabolic activation, and in vivo, methods for induction of tolerance to selenium and to alkylating agents (such as by heavy metal exposure [80,81]) and thiol modulation of normal tissue toxicities.

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