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Nucleophilic Selectivity Ratios of Model and Clinical Alkylating Agents by 4-(4′-Nitrobenzyl)pyridine Competition

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SUMMARY

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An aqueous acetone 4-(4′-nitrobenzyl)pyridine (NBP) assay for alkylating activity was used at neutral pH to reproduce the Swain-Scott-Ogston reactivity sequence of nucleophile n constants, based on bimolecular competition between NBP and added nucleophiles for alkylation by nitrogen mustards, methanesulfonate esters, methyl iodide, epichlorohydrin, or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Nucleophile concentration-dependent decreases in the 540-nm NBP product(s) permitted determination of the second-order rate constant ratio, k_x/k_{nbp}, for alkylation of the competing nucleophile/NBP. The slope, s^*, of several $\log(k_x/k_{nbp})$ values versus n constants closely paralleled literature Swain-Scott s constants and could be derived from the value for thiosulfate competition, $\log(k_{s_2o_3}/k_{nbp})$. Alkylating activity, defined by the maximal 540-nm absorbance found for reactions up to 1 hr, was clearly related to an alkylating agent's nucleophilic sensitivity [s, s^*, or $\log(k_{s_2o_3}/k_{nbp})$ value]. Values of $\log(k_{s_2o_3}/k_{nbp})$ corresponding to s constants 0.78–1.39 for clinical alkylating agents increased in the order 1-chloroethyl-3-(4-methylcyclohexyl)-1-nitrosourea ~ streptozotocin < 1,4-bis(methanesulfonoxy)butane ~ BCNU < 1,1′,1″-phosphinothioylidynetrisaziridine ~ methyl-bis(2-chloroethyl)amine < 4-[bis(2-chloroethyl)amino]-L-phenylalanine. NBP competition methods for determination of nucleophilic selectivity ratios should be highly useful for rapid identification of protective nucleophile-clinical alkylating agent combinations, and for classification of alkylating agent biological mechanism of action according to recent interpretations of alkylating agent kinetic behavior.

INTRODUCTION

The reactivity of an alkylating agent is often defined by its time-dependent alkylation of NBP[2] in the presence of competing nucleophiles such as buffer and solvent molecules (1, 2). The reactivity of NBP toward alkylating agents results from the relatively strong nucleophilicity of the anionoid (3) electron pair of the pyridine ring nitrogen. The nucleophilicity order described for sulfur mustard in water (4) is essentially the same order as that reported for nitrogen mustards (5) and for primary (sterically unhindered, saturated) carbon electrophilic centers in protic solvents (6–8):

$$S_2O_3^{2-} > HS^- > \text{pyridine} > SO_4^{2-} > NO_3^- > H_2O$$

The reactivities within this nucleophilic series are determined by polarizability factors and to a lesser extent, by basicity strength; molar refractivities of nucleophiles parallel their reactivities as do electrode potentials (9). There is also a resemblance of the nucleophilicity order to the lyotropic (Hofmeister) anion series for salting proteins out of solution (10). Increased hydrogen bonding and greater solvation energies accompany higher electron localization of the weaker nucleophiles in solvents with

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[2] The abbreviations used are: NBP, 4-(4′-nitrobenzyl)pyridine; EPI, epichlorohydrin; MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; HN2, methyl-bis(2-chloroethyl)amine hydrochloride; nor-HN2, bis(2-chloroethyl)amine hydrochloride; chlorambucil, 4-[p-[bis(2-chloroethyl)amino]phenyl]-butyric acid; L-PAM, 4-[bis(2-chloroethyl)amino]-L-phenylalanine; busulfan, 1,4-bis(methanesulfonoxy)butane; streptozotocin, 2-deoxy-2-[[(methylnitrosoamino)-carbonyl]amino]-D-glucopyranose; MeCCNU, 1-chloroethyl-3-(4-methylcyclohexyl)-1-nitrosourea; BCNU, 1,3-bis(2-chlorethyl)-1-nitrosourea; thioTEPA, 1,1′1″-phosphinothioylidynetrisaziridine; HSM, 2-chloroethyl-(2′-hydroxyethyl)sulfide; PM·CHA, *N,N*-bis(2-chloroethyl) phosphorodiamidic acid, cyclohexylamine salt; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MEA, 2-mercaptoethylamine; MNU, methylnitrosourea.

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high dielectric constants. Thus, poorly polarizable reactants in polar solvents are rendered less nucleophilic by solvation energy barriers which contribute to, or perhaps cause, the normally wide range of nucleophilic reactivities.

Alkylating agents show exponential variation in the degree of their discrimination among nucleophiles in protic solvents. This property, alkylating agent nucleophilc selectivity, was placed on a quantitative basis by Swain and Scott (6) soon after the original nucleophilicity order was published by Ogston *et al.* (4) in 1948. The nucleophilic selectivity of an alkylating species is most simply defined as the ratio of second-order rate constants, k_x/k_y, for the independent, competitive reactions of the electrophilic species that alkylates nucleophiles X and Y, under pseudo-first-order conditions of excess nucleophile concentrations (11, 12).

Recently, it has become apparent that variation in an alkylating agent's nucleophilic selectivity is a major determinant of initial patterns of intracellular alkylation of biological macromolecules. In particular, it has been postulated that relatively low nucleophilic selectivity may result in greater alkylation of weakly nucleophilic oxygen substituents in nucleic acids and in enhanced mutagenic efficiency (12, 13). Conversely, extremely poor discrimination among biological nucleophiles may result in hydrolysis of the alkylating species prior to diffusion to target nucleophile(s) (14), thus quenching its biological effects. The extremely fast reactions of antitumor mustard-type alkylating agents has until now precluded a meaningful comparison of their nucleophilic selectivities. Nevertheless, Ross (5) early recognized the potential importance of nucleophilic selectivity in clinical alkylating agent drug design by suggesting that the high nucleophilic selectivities of mustards caused suboptimal intracellular alkylation due to biologically irrelevant alkylation of strongly nucleophilic groups during transport.

NBP-derived alkylating activities of various antineoplastic agents are well known to correlate with drug toxicities and occasionally, with antineoplastic effects (2, 15). However desirable, few conclusions regarding an alkylating agent's nucleophilic selectivity can be drawn from published NBP reactivity values, because of the varying NBP reaction conditions employed, and because of the unpredictability of reactivity-selectivity relationships (11). Pross (11), a Swedish group (12), and others (16) have emphasized the useful fact that nucleophilic selectivity, the ratio of second-order rates k_x/k_y, can be calculated from P_x/P_y, the ratio of alkyl products formed:

$$\frac{k_x}{k_y} = \frac{(P_x)[Y]}{(P_y)[X]} \tag{1}$$

where $[X]$ and $[Y]$ are the initial nucleophile concentrations. Since nucleophilic displacement reactions at primary carbon electrophilic centers may be expected to follow bimolecular kinetics (16), a rate-product correlation is expected upon addition of a nucleophilic salt to the solvolytic reaction medium (17). Bimolecular kinetics are followed in the NBP assay of "classical" alkylating agents, since the rate of formation of product chromophore is first-order in NBP as well as in alkylating agent concentration (2, 18). If it is assumed that a decrease in absorbance in the NBP assay, which occurs on addition of a nucleophile, X, is entirely a result of new alkyl product formed, then

$$\frac{P_x}{P_y} = \frac{A_c}{A_0} - 1 \tag{2}$$

where P_x/P_y is the ratio of new alkyl product (P_x) to product chromophore(s) of NBP alkylation (P_y), and A_c/A_0 is the control absorbance divided by the absorbance with added competing nucleophile. It follows that

$$\log\left(\frac{k_x}{k_y}\right) = \log\left(\frac{[Y]}{[X]}\left[\frac{A_c}{A_0} - 1\right]\right) \tag{3}$$

Equation 3 is related to the Swain-Scott (6) linear free energy relationship (Eq. 4), whereby the nucleophilic selectivity of an alkylating agent is defined as the slope, s, of second-order rate constants for nucleophilic substitution plotted against standard relative rates (expressed as n constants for different nucleophiles) for reaction with the reference substrate CH_3Br

$$\log(k_x/k_y) = s(n_x - n_y) \tag{4}$$

The intrinsic nucleophilic strengths of reagents X and Y for attack on CH_3Br are given by the constants n_x and n_y. By convention, the intrinsic electrophilicity constant (the degree of selectivity among nucleophiles by the alkylating agent), s, equals 1.00 for CH_3Br. When Y is H_2O, $n_y = 0.00$, and the familiar expression $\log(k_x/k_0) = sn_x$ results where k_0 is the second-order hydrolysis constant. Thus, the logarithm of the second-order rate constant ratio, $k_{s_2o_3}/k_0$, describing thiosulfate competition with water for alkylation by CH_3Br is 6.36, which is the n constant for thiosulfate (4, 6, 8). Log $(k_{s_2o_3}/k_0)$ is a surprisingly clear predictor of s (using several nucleophiles) for nitrosoureas, methanesulfonate esters, nitrogen and sulfur mustards, as well as for traditional Swain-Scott substrates such as EPI (5–8, 12, 13, 19), whether the slopes of second-order rates versus n constants represent basic or anionic nucleophiles (7, 12). However, use of hydrolysis rate denominators has been criticized (8) because of the dual role of solvent, both as nucleophile and electrophile. NBP is weakly basic, with a pK_a of 3.51 (this report). The large difference in nucleophilicities of thiosulfate and NBP, taken together with their predictably weak leaving group constants (20) and weak basicities, therefore suggested use of thiosulfate competition in the NBP assay for rapid estimation of s for a wide variety of alkylating agents, including several examples of therapeutic, mutagenic, and Swain-Scott alkyl substrates.

MATERIALS AND METHODS

Chemicals. Alkylating agents were obtained from the following sources and used in the solvents indicated: MMS, EMS, iodoacetic acid, benzyl chloride, and EPI, Eastman Organic Chemicals, Rochester, N. Y. (acetone); methyl iodide, Aldrich Chemical Company, Milwaukee, Wisc. (acetone); ethylene oxide, Pfaltz & Bauer Inc., Stamford, Conn. (acetone); HN2 and nor-HN2, Aldrich Chemical Company (water); chlorambucil (ether), L-PAM (ethanol), and busulfan (acetone), Burroughs-Well-

come, Research Triangle Park, N. C.; streptozotocin (water) and MeCCNU (dimethyl sulfoxide), the Division of Cancer Treatment, National Cancer Institute; BCNU (ethanol) and mitomycin C (water), Bristol Laboratories, Syracuse, N. Y.; thioTEPA (acetone), Lederle Laboratories, Wayne, N. J.; HSM (ether), Dajac, Philadelphia, Pa. PM·CHA (water) was a gift of Dr. T. C. Hall, and MNNG (acetone) was kindly supplied by Dr. A. R. Peterson of the University of Southern California Cancer Center. NBP was an Aldrich product. Folinic acid (calcium leukovorin) was obtained from Lederle, and L-1-(+)-tetrahydrofolate was prepared as described previously (21). All other reagents were commercially available reagent grade and were used as received.

NBP nucleophilic selectivity assay. A modification of the Friedman and Boger procedure (1) was used. In sequence, 1 ml of H_2O, 0.2 ml of Tris-HCl buffer (0.2 M, pH 7.0), and 1 ml of 2% (w/v) solution of NBP in acetone were added at 10° in 13 × 100 mm borosilicate air-tight screw-cap test tubes, and the solution was Vortexed to a single phase; 0.1 ml of the alkylating agent (≤1.0 mM in the appropriate solvent) was added. The tubes were tightly stoppered, mixed, shielded from ambient light, and placed in a 95° water bath for periods of time corresponding to 3–10 half-lives of the NBP alkylation reaction, up to 60 min. The reaction mixtures were cooled in an ice-water bath, in which the NBP chromophore was stable for up to 2 hr. To the mixtures at 20° were added 1 ml of acetone, 2.5 ml of ethyl acetate, and 1 ml of 0.25 N NaOH; the tubes were inverted 20 times and centrifuged for 45 sec at 1300 × *g*. The $E^{540}_{1\,cm}$ of the upper, organic phase, was determined in a Beckman Model 25 spectrophotometer exactly 3 min after alkalinization. A blank for each determination was obtained from paired reaction mixtures without alkylating agent, or without NBP.

A_c values were obtained from the standard NBP assay. Paired A_0 values were obtained by adding the competing nucleophile, over a several log concentration range, into the NBP assay in the 0.2 M Tris-HCl buffer at pH 7.0. Of the nucleophiles studied, only pyridine and Na_2SO_3 required pH adjustment, which was done with glacial acetic acid (at concentrations below measurable effects on NBP absorbance). Only sodium salts of anions were used. The measured incubation pH in all cases was between 6.5 and 7.0, before or after heating, using a Beckman 3550 pH meter. Control A_c values over this pH range did not vary beyond experimental error, and gave absorbances at least as great as the standard NBP assay (1) which uses acetate buffer. Values of A_0 between 5% and 95% of A_c and which occurred between a 4-fold and 1000-fold molar excess of added nucleophile over the initial alkylating agent concentration were used for nucleophilic selectivity calculations by Eq. 3.

Statistics. Linear regression analyses were done by use of standard SR-51-II and TI-55 programs and standard N-2 significance tables.

RESULTS

Nucleophilic reactivity order. HN2 was chosen initially as the standard alkylating agent because of its predictably high selectivity among nucleophiles (5, 12), its high reactivity, and broad solubility characteristics. Reagents with established Swain-Scott *n* values were used for nucleophilicity assay by their concentration-dependent inhibition of formation of the 540-nm HN2-NBP product(s). Decreases in absorbance at 540 nm were found to occur at nucleophile concentrations logarithmically related to their *n* constants. Representative data from individual runs for five nucleophiles are shown in Fig. 1. The sigmoidal curves of absorbance inhibition caused by increasing concentrations of added nucleophile followed bimolecular kinetics calculated by Eq. 3 within experimental error. Percentage decrease curves for $S_2O_3^{2-}$ and N_3^- did not change with log-fold variation in HN2 concentration below competing nucleophile concentration. Chloride ion's absorbance decreases [$\log(k_{cl}/k_{nbp}) = -1.03 \pm 0.06$] were in surprisingly good agreement with its *n* value (Fig. 2). The concentration of Tris-HCl (17.4 mM) was ≤5% of its concentration causing a 50% decrease in the NBP product. Heat treatment at 95° of alkylating agent-free NBP assay mixtures containing 0.80 mM [$S_2O_3^{2-}$] caused a decrease of ≤0.10/hr in $\log(k_{s_2o_3}/k_{nbp})$ value for subsequent HN2 addition, which effect was ignored in $\log(k_{s_2o_3}/k_{nbp})$ calculation for more slowly reacting alkylating agents.

The results of $\log(k_x/k_{nbp})$ values derived from Eq. 3 were correlated with literature *n* constants for 10 nucleophiles in competition with NBP for attack on HN2, as summarized in Table 1. The individual data are shown in Fig. 2. The standard deviations in Fig. 2 reflect the experimental error found for the 5%–95% absorbance decrease range, and represent the average results of two or more runs per nucleophile. The order of nucleophilic reactivities for HN2, spanning a 100,000 fold range, from the powerfully nucleophilic thiosulfate ion [$\log(k_{s_2o_3}/k_{nbp})$

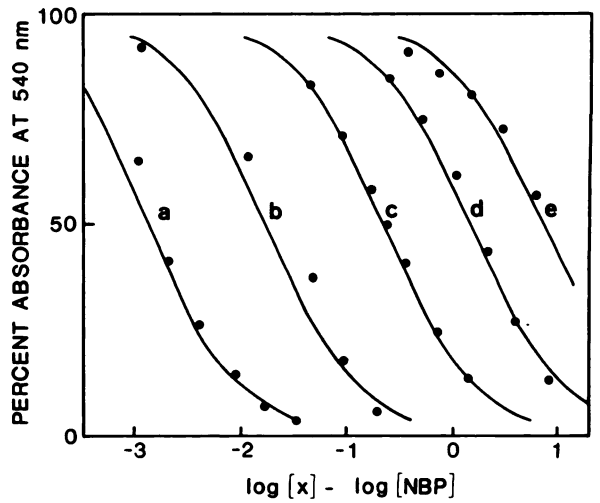

FIG. 1. *Nucleophile concentration versus absorbance decrease in the NBP assay of HN2*

Representative single runs shown were the result of 10-min incubations of mixtures at 95° containing 40.6 mM NBP, 0.043 mM HN2, 17.4 mM Tris-HCl (pH 7.0), 52% (v/v) aqueous acetone, and varying concentrations of competing nucleophile, *X*: *a*, [$S_2O_3^{2-}$]; *b*, [SO_3^{2-}]; *c*, [N_3^-]; *d*, [pyridine]; *e*, [CH_3COO^-]. *Solid lines* show the predicted curvature of simple bimolecular kinetics between NBP and an added nucleophile for alkylation by HN2. Data points of the absorbance profiles were used for Eq. 3 calculation of second-order rate constant ratios, $\log(k_x/k_{nbp})$ values.

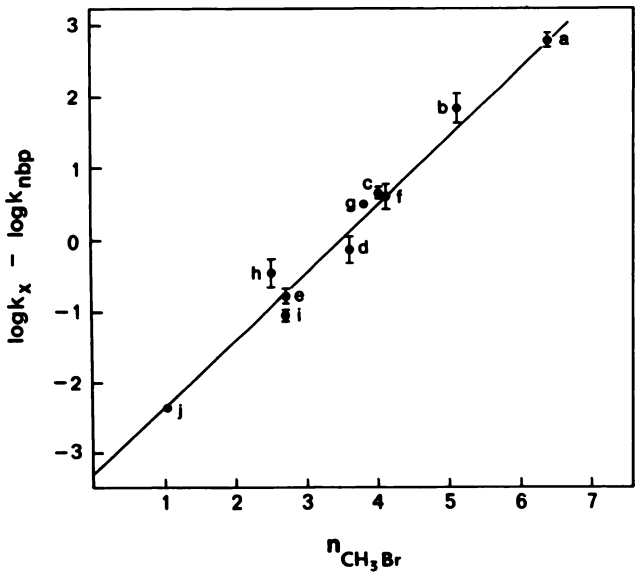

FIG. 2. *Relationship of log(k_x/k_{nbp}) values for 10 nucleophiles in reaction with HN2, to Swain-Scott-Ogston nucleophilic reactivity n constants for alkylation by CH_3Br (refs. 4, 6, 8)*

At constant (40.6 mM) NBP concentration for all nucleophiles studied, log(k_x/k_{nbp}) is equal by Eq. 3 to log(40.6/[X]) at that concentration of added nucleophile, [X], which causes a 50% absorbance decrease: *a*, [$S_2O_3^{2-}$]; *b*, [SO_3^{2-}]; *c*, [N_3^-]; *d*, [pyridine]; *e*, [CH_3COO^-]; *f*, [$(NH_2)_2CS$]; *g*, [HPO_4^{2-}]; *h*, [SO_4^{2-}]; *i*, [Cl^-]; *j*, [NO_3^-]. The linear correlation (see Eq. 5 and Table 1) showing the high nucleophilic selectivity of HN2 ($s^* = 0.983$) was used for derivation of n (~3.5) for NBP, and permitted measurement of n for new nucleophiles such as reduced folates (see Results).

= 2.81] to the poorly reactive nitrate ion [log(k_{no_3}/k_{nbp}) = −2.35], accurately reproduced the sequence of literature n constants:

$$0.983n = \log(k_x/k_{nbp}) + 3.391 \quad (5)$$
$$(r = 0.9871;\ N = 10;\ p < 0.001)$$

This was expected because the correlation between the logarithm of Ogston's "competition factor" characterizing nucleophiles for sulfur mustard (4) and Swain-Scott n constants (6) is nearly perfect ($r = 0.9969$) for the 11 nucleophiles common to those studies (omitting OH^-, these were $HPSO_3^{2-}$, $S_2O_3^{2-}$, HS^-, I^-, NCS^-, $(NH_2)_2CS$, HCO_3^-, HPO_4^{2-}, Cl^-, CH_3COO^-, and SO_4^{2-}). The competition factor of a nucleophilic reagent for sulfur mustard was historically defined as "the ratio of the rate of its reaction to that of water, when the concentration of the reagent is 1 equivalent per liter" (22). The present approach, instead, has varied the added nucleophile concentration in order to achieve a competing rate of alkylation similar to the NBP alkylation rate, assumed to be a constant. Hydrolytic rates were not assayed in this study, but the effects of hydrolysis on nucleophilic selectivity determinations by log($k_{a_2o_3}/k_{nbp}$) measurement do become significant for poorly selective alkylating agents, discussed below and in the Appendix.

The very good (8) correlation shown by Eq. 5 (Fig. 2) is in accord with the assumption that the NBP product method gives second-order rate ratio, kinetic (n constant) results; this is attributable in part to the stability of the products, and to the expected general correspondence between rates in the product-forming reactions and the product spread for primary alkyl substrates (11, 16, 17). High acidity in the nucleophile facilitates leaving group behavior (20) and therefore secondary displacement on the alkyl-nucleophile product. Significant secondary NBP alkylation by nucleophile product(s) should cause increasingly negative deviations in log(k_x/k_{nbp}) values with increasing nucleophile concentration, unlike the result shown in Fig. 1. Prolonged heating (90 min) in the case of NO_3^-, however, was found to lead to increases in alkylating activity approximately 10% above A_c values, with consequent decreases in log(k_x/k_{nbp}) values, possibly due to secondary NBP alkylation by HN2-nitrate(s). With shorter heating periods, no apparent difference between nonbasic [$S_2O_3^{2-}$, $(NH_2)_2CS$, SO_4^{2-}, Cl^-, NO_3^-] and basic (SO_3^{2-}, N_3^-, HPO_4^{2-}, pyridine, CH_3COO^-) nucleophiles is evident in the correlation between log(k_x/k_{nbp}) and n constants for HN2 (Fig. 2). Low acidity, with

TABLE 1

Nucleophilic selectivities of model and clinical alkylating agents

Slopes (s^*) of linear regression correlations between log(k_x/k_{nbp}) values and Swain-Scott-Ogston nucleophilicity n constants[a]: $s^*n = \log(k_x/k_{nbp}) - \log(k_0/k_{nbp})$.

Alkylating agent	Nucleophiles (X) studied for log(k_x/k_{nbp}) value	s^*	log(k_0/k_{nbp})[b]	r	p	Literature s (ref.)
HN_2	$S_2O_3^{2-}$, SO_3^{2-}, $(NH_2)_2CS$, N_3^-, HPO_4^{2-}, pyridine, SO_4^{2-}, CH_3COO^-, Cl^-, NO_3^-	0.983	−3.391	0.9871	<0.001	1.13[c] (19)
CH_3I	$S_2O_3^{2-}$, NCS^-, N_3^-, NO_3^-	0.926	−3.418	0.9835	<0.02	1.23 (7)
EPI	$S_2O_3^{2-}$, NCS^-, N_3^-, NO_3^-	0.753	−3.390	0.9982	<0.01	0.93 (6)
MMS	$S_2O_3^{2-}$, NCS^-, N_3^-, NO_3^-	0.717	−3.101	0.9818	<0.02	0.86 (12)
EMS	$S_2O_3^{2-}$, HPO_4^{2-}, pyridine, Cl^-, NO_3^-	0.606	−2.737	0.9907	<0.001	0.67 (12)
BCNU	$S_2O_3^{2-}$, SO_3^{2-}, NCS^-, N_3^-, HPO_4^{2-}, pyridine, Cl^-	0.717	−2.799	0.9473	<0.01	
PM·CHA	$S_2O_3^{2-}$, SO_3^{2-}, N_3^-, MEA, ascorbate	0.953	−3.527	0.9915	<0.001	
L-PAM	$S_2O_3^{2-}$, N_3^-	1.186	−4.146	—	—	

[a] Values for n constants were taken to be 6.36 ($S_2O_3^{2-}$), 5.1 (SO_3^{2-}), 4.77 (NCS^-), 4.1 ($[NH_2]_2CS$), 4.0 (N_3^-), 3.8 (HPO_4^{2-}), 3.6 (pyridine), 2.72 (CH_3COO^-), 2.7 (Cl^-), 2.5 (SO_4^{2-}), and 1.03 (NO_3^-), refs. 4, 6, 8; n constants for MEA and ascorbate were 3.4 and 3.3 (this study). Log(k_x/k_{nbp}) values were calculated by Eq. 3 (where Y = NBP) as described under Materials and Methods. At constant (40.6 mM) NBP concentration in the NBP assay, (k_x/k_{nbp}) is equal to the concentration ratio [40.6]/X at which a 50% absorbance decrease is found to occur.

[b] Intercept values represent the theoretical second-order rate ratio (k_0/k_{nbp}) of water/NBP at $n = 0.00$.

[c] Value for *N,N*-diethylethylenimonium ion.

the exception of the halides, is characteristic of the Ogston nucleophiles, which had been selected for "competition therapy" potential (4, 5, 22) and therefore for anticipated stability in the alkyl products.

The n constant of NBP can be calculated from the Eq. 5 relationship for HN2. At $n = 0.00$, k_x represents the hydrolysis constant, k_0, so that n of NBP is approximately 3.5. This is not significantly different from the n constant for pyridine which is about 3.6 (4). Equation 5 also permitted study of nucleophiles of unknown n constants for which bimolecular, sigmoidal curves of absorbance decreases in the NBP assay of HN2 occurred with increasing concentration of nucleophile. According to Eq. 5, experimentally derived n constants for Tris-HCl, MEA, and sodium ascorbate were 2.3 ± 0.2, 3.4 ± 0.2, and 3.3 ± 0.1, respectively. The n constants found for L-1-(+)-tetrahydrofolate and folinic acid were identical at 5.4 ± 0.1, which result may suggest that the N-10 site is the strongest nucleophilic center for these folates.

Alkylating agent selectivity. The slope of the regression line given by Eq. 5 shown in Fig. 2 directly gives the apparent Swain-Scott nucleophilic selectivity value, s^* (to be distinguished from previously published s constants), equal to 0.983 for HN2. This value is somewhat lower than the s constant found by Jones *et al.* (19) of 1.13 for the *N,N*-diethylethylenimonium ion. In order to determine the relationship of the present s^* value obtained by varying ratios of nucleophile concentrations versus the traditional approach of taking rate ratios at similar nucleophile concentrations, s^* was determined for other alkylating agents with well-established literature s constants in protic solvents. These were CH_3I ($s = 1.23$, ref. 7); EPI ($s = 0.93$, ref. 6); MMS ($s = 0.86$) and EMS ($s = 0.67$) (12). The nucleophiles used in s^* determination were mostly nonbasic: $S_2O_3^{2-}$, NCS^-, N_3^-, NO_3^- (CH_3I, EPI, MMS); $S_2O_3^{2-}$, HPO_4^{2-}, pyridine, Cl^-, and NO_3^- (EMS). The resulting correlation between s^* (listed in Table 1) and published s constants for the five alkylating agents was

$$s = 1.365s^* - 0.124 \quad (r = 0.9542;\ N = 5;\ p < 0.02) \qquad (6)$$

If zero intercepts are assumed, $s = 1.234\ s^*$. Alkylating agents for which no previously published s constant could be found were studied for s^* determination: L-PAM, BCNU, and PM·CHA. Table 1 lists the nucleophiles used and the resulting s^* values. No significant differences in s^* occurred on omission of the basic nucleophiles, but only the calculated concentrations of the divalent species of phosphate and sulfite, and of unprotonated pyridine were considered (from pK_a at 20° in H_2O at pH (6.8).

For reasons given, and to standardize and simplify nucleophilic selectivity determinations, correlations were sought and found between the results of thiosulfate concentration changes in the NBP assay, $\log(k_{s_2o_3}/k_{nbp})$ values alone, and published s constants or the present s^* values. The results are shown in Fig. 3. Since the derived n of NBP is 3.5, under the conditions of this study, it might have been expected based on Eq. 4 that

$$\log(k_{s_2o_3}/k_{nbp}) = s^*(6.36 - 3.5) = 2.86s^* \qquad (7)$$

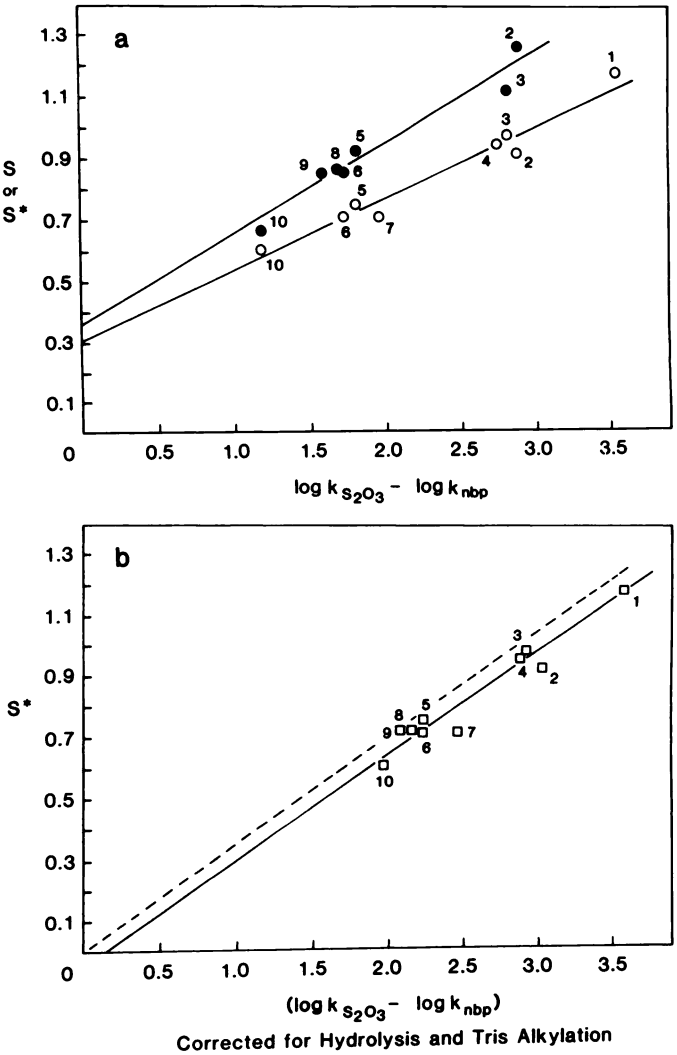

FIG. 3. *Nucleophilic selectivity determination by changing thiosulfate concentration in the NBP assay of 10 alkylating agents*

Charts show the linearity between alkylating agents' published Swain-Scott s constant (●) or present s^* value (○) and the second-order rate constant ratio, $\log(k_{s_2o_3}/k_{nbp})$ value by Eq. 3 in *panel a* and after correction (□) for bimolecular hydrolysis and Tris alkylation in *b*, discussed in Appendix (Eq. 16). Alkylating agents were: *1*, L-PAM; *2*, CH_3I; *3*, HN2 and the *N,N*-diethylethylenimonium ion; *4*, PM·CHA; *5*, EPI; *6*, MMS; *7*, BCNU; *8*, benzyl chloride; *9*, ethylene oxide; and *10*, EMS. The s^* values of benzyl chloride and ethylene oxide in *b* were calculated by Eq. 6. The results in *b* were anticipated by Eq. 7, shown by - - -, based on the HN2-derived nucleophilicity of NBP.

However, these approximately linear relationships (Fig. 3a) were obtained:

$$s = 0.285 \log(k_{s_2o_3}/k_{nbp}) + 0.381 \quad (r = 0.9811;\ N = 7;\ p < 0.001) \qquad (8)$$

and

$$s^* = 0.237 \log(k_{s_2o_3}/k_{nbp}) + 0.304 \quad (r = 0.9814;\ N = 8;\ p < 0.001) \qquad (9)$$

The ratio of the slopes of Eqs. 8 and 9 (1.20) points up the somewhat lower s^* values compared to literature s constants. Both slopes similarly demonstrate that the

less selective agents show increasingly negative deviations from their expected (Eq. 7) $\log(k_{s_2o_3}/k_{nbp})$ values. These deviations are clearly a result of the increasing role of water as a competing nucleophile for decreasingly selective alkylating agents. Incorporation of a term for the concentration of water into Eq. 7 makes the *a priori* prediction that at zero observed $\log(k_{s_2o_3}/k_{nbp})$, the calculated s constant will be 0.45, which is close to the Eq. 8 experimental result. However, s^* determinations by use of two or more nucleophiles' $\log(k_x/k_{nbp})$ values, should not in a likewise manner be affected by hydrolysis and other competing side reactions such as alkylation of the Tris buffer (see Appendix for discussion), which explains the similar intercepts of Eqs. 8 and 9 and explains the Fig. 3b demonstration of the simple correspondence between s^* and "corrected" $\log(k_{s_2o_3}/k_{nbp})$ values.

The finding of consistently lower s^* values as compared with s constants is probably due to complex positive salt effects, caused in part by the large range of nucleophilic salt concentrations required for $\log(k_x/k_{nbp})$ determination via Eq. 3. Most of the nucleophilic salts of the present study are likely to linearly increase first-order rates of alkyl substrate consumption beyond that expected for alkylation of the salt per se, yet quantitative separation of such effects from simple bimolecular alkylation of the added salt proves exceedingly difficult (7, 17, 19). Furthermore, weakly nucleophilic protonated buffer molecules have a catalytic role in NBP alkylation by mustards (1, 18). No evidence for lowering of nucleophilic selectivity by the high temperature of the present study was obtained by repeating measurements of $\log(k_{s_2o_3}/k_{nbp})$ values of L-PAM, HN2, nor-HN2, CH_3I, MMS, and EMS at 20°, for long incubation times (up to 7 days in the dark). In fact, all of the latter values (not shown) were approximately 0.3 log unit lower than their 95° values, and their control (no added nucleophile) absorbances or alkylating activities were likewise decreased about 50%.

The nucleophilic selectivity ratio, $\log(k_{s_2o_3}/k_{nbp})$, for 21 alkylating agents (all of which gave absorbance peaks at 540 nm) is presented in Table 2, together with Eq. 8 derived s constants and relative alkylating activity. The derived s constant of thioTEPA is 1.10, slightly higher than the 0.90 value reported (23) for simpler aziridines. The result for MNNG may be compatible with published data (ref. 13, and references cited therein) for several nucleophiles, which, however, show large enough deviations from the normal nucleophilic order (in part because MNNG possesses two asymmetrical electrophilic sites) that choice of a suitable literature s constant is difficult. The most serious deviation occurs for MNU, which has a reported s of 0.42 (13) and a calculated s of 0.71, which could be in error because of the extremely low alkylating activity of MNU (or alternatively, because of temperature- or nucleophile-related effects on its mechanism(s) of nucleophilic substitution).

The significance of alkylating activity is that it is another parameter of nucleophilic selectivity. This is shown by the parallelism between $\log(k_{s_2o_3}/k_{nbp})$ values and alkylating activity (Table 2). Furthermore there is a correspondence between values of $\log(k_0/k_{nbp})$ (Table 1) and nucleophilic selectivity parameters. The ratio (k_0/k_{nbp}) represents the theoretical ratio of NBP to water, which will cause a 50% decrease in alkylated NBP product due to bimolecular competing hydrolysis (and which allowed calculation of the n constant of NBP, above). Direct determination of $\log(k_0/k_{nbp})$ was not possible. Although NBP absorbance does increase in a sigmoidal, bimolecular fashion with increasing NBP concentration, and the 40.6 mM NBP assay concentration was on the "plateau" portion of the curve for HN2, at [NBP] ≥200 mM, paradoxical absorbance increases were observed in the extracted colorimetric product. Variation in $[H_2O]$ was limited by the insolubility of NBP in water, and also by water's over-all rate-accelerating effect. Therefore, alkylating activity is expressed simply as percentage of the absorbance found in the NBP assay for equimolar HN2.

TABLE 2

Nucleophilic selectivity of alkylating agents by thiosulfate-mediated decreases in the NBP assay

Alkylating agent	AA[a]	Log$(k_{s_2o_3}/k_{nbp})$[b]	Derived[c] s
L-PAM	110%	3.54 ± 0.08	1.39
Chlorambucil	100%	3.10 ± 0.21	1.26
Nor-HN2	100%	3.08 ± 0.06	1.26
CH_3I	62%	2.88 ± 0.15	1.20
HN2	100%	2.81 ± 0.12	1.18
PM·CHA	62%	2.75 ± 0.10	1.16
HSM	—	2.69 ± 0.03	1.15
ThioTEPA	80%	2.53 ± 0.17	1.10
ICH_2COOH	75%	2.51 ± 0.11	1.10
BCNU	50%	1.95 ± 0.12	0.94
Busulfan	20%	1.81 ± 0.13	0.90
EPI	42%	1.80 ± 0.06	0.89
MMS	60%	1.72 ± 0.05	0.87
Benzyl chloride	40%	1.67 ± 0.08	0.86
Ethylene oxide	5%	1.58 ± 0.05	0.83
MNNG	1%	1.55 ± 0.18	0.82
Mitomycin C	13%	1.52 ± 0.21	0.81
Streptozotocin	3%	1.43 ± 0.10	0.79
MeCCNU	2%	1.39 ± 0.04	0.78
EMS	15%	1.17 ± 0.02	0.71
MNU	2%	1.14 ± 0.04	0.71

[a] Alkylating activity as percentage of equimolar HN2 540-nm absorbance at ≥3 half-lives of the observed alkylation rate, up to 60 min.

[b] Represents the average ± standard deviation of five data points per run and two runs per alkylating agent, based on Eq. 3.

[c] Directly calculated from $\log(k_{s_2o_3}/k_{nbp})$ values by Eq. 8 shown in Fig. 3a.

DISCUSSION

Since it is likely that intracellular reactivities of nucleophiles correspond to their chemical reactivities (5, 12, 13), the broad range of nucleophilic selectivities found in this study for the clinical alkylating agents implies necessarily varied biological mechanisms of action. Many biologically important nucleophiles such as sulfhydryl, phosphate, bicarbonate, and imidazole groups are intrinsic to the Swain-Scott-Ogston nucleophilic reactivity order. The strongest nucleophile known is a reduced form of vitamin B_{12} that has an n constant of ~10.3 (24). Duplex, native DNA is perhaps 20-fold more nucleophilic

than monomeric DNA (25) and thus may have a calculable n constant of ~5.2 (4, 13, 25). The present finding of $n = 5.4$ for reduced folates suggests that these are also important intracellular alkylating agent targets.

The preferential reaction of an alkylating agent with the stronger nucleophiles relative to weaker ones, when both are present in excess, will rise steeply with increasing nucleophilic selectivity, i.e., with increasing Swain-Scott s constant. The slopes of $\log(k_x/k_{nbp})$ values for two or more nucleophiles plotted against their literature n constants, i.e., s^* values (Table 1), closely paralleled literature s constants for CH_3I, EPI, MMS, EMS, and the ethylenimonium ion (Eq. 6). The s^* values were slightly lower than s constants, most likely due to complex positive salt effects caused by the high concentrations of nucleophiles needed for s^* determination. It may be that high nucleophile concentrations have subtle "solvent sorting" effects on nucleophilic selectivity. For example, preliminary results with HN2 have suggested a lowering of $\log(k_{s_2o_3}/k_{nbp})$ values in the presence of half-molar concentrations of NBP and $NaNO_3$.

More conveniently, and to avoid the potential problems of the higher concentrations used for weaker nucleophiles, $\log(k_{s_2o_3}/k_{nbp})$ values for thiosulfate competition alone were correlated with s and s^*. Although the effect of competing hydrolysis caused a calculable, wider spread of $\log(k_{s_2o_3}/k_{nbp})$ values than expected (see Fig. 3 and Appendix), strongly linear relationships (Eqs. 8 and 9) were found for prediction of s or s^* by the sole use of thiosulfate addition to the NBP assay.

A great advantage of the present NBP product competition approach for determination of s^* is that the concentration of alkylating species need not be precisely known, as is necessary for determination of second-order rate ratios by traditional kinetic methods. NBP alkylating activity (below the limiting value of 100%) itself may give some approximation of nucleophilic selectivity; however, the extinction coefficient of the alkylated NBP chromophore will depend on the nature of the alkyl moiety, although this is perhaps not significant if substituent effects within a single class of alkylating agents are considered (2, 18).

Observed first-order rates of NBP alkylation have not been included in the present study but have been used in the past for comparison of the relative tendencies of aromatic and aliphatic nitrogen mustards to undergo bimolecular substitution mechanisms of reaction (2). Such an approach has limited utility, because in many cases the observed NBP alkylation rate will only reflect the slowness of the initial ionization step in forming the reactive aziridinium intermediate (4, 5, 25).

Table 2 lists the nucleophilic selectivity parameters $\log(k_{s_2o_3}/k_{nbp})$, calculated s constant, and alkylating activity of 21 alkyl substrates for nucleophilic substitution reaction. It should be noted that the selectivity of an alkylating agent for the thiosulfate anion does not always predict for reaction rates with other model nucleophiles. For example, the triphenylmethyl halides, which probably react through a tertiary carbonium ion intermediate, react faster with azide ion than with thiosulfate (26). However, with the exception of MNNG and mitomycin C (which is a 1,2-disubstituted aziridine) all alkyl substrates in the present report (Table 2) are primary, saturated carbon electrophiles which are expected to follow the normal nucleophilic order.

The NBP product competition method for determination of Swain-Scott s and n constants is ideally suited for the screening of nucleophiles for their potential "protective" effect against a given alkylating agent. Ogston's nucleophilicity series was in fact studied for the purpose of identifying antidotes for sulfur mustard toxicity (4, 5, 22). The n constant for small molecular weight nucleophiles is readily determined from their relative log (k_x/k_{nbp}) values, using a standard substrate such as HN2 (shown in Fig. 2). The high nucleophilicity of the clinically useful folinic acid, for example, makes this a particularly interesting "protective" candidate. Concentration-dependent protection by thiols against alkylating agent cytotoxicity or against DNA alkylation *in vitro* has repeatedly been demonstrated for highly selective alkylating agents such as L-PAM and HN2, but has been less convincingly shown for busulfan (27), which is less selective among nucleophiles (Table 2).

The clinical alkylating agents vary so greatly in their nucleophilic selectivities that nearly the entire range of published s constants is represented. The mustards demonstrate nucleophilic selectivities that are comparable to or greater than that of model sulfhydryl-inhibiting monofunctional alkylating agents, such as iodoacetic acid. In contrast, streptozotocin and MeCCNU show nucleophilic selectivities similar to that of model mutagenic and carcinogenic alkylating agents, such as MNNG, EMS, and MNU. Potentially the most important observation in this study is that nucleophilic selectivity values for the most selective clinical alkylating agents have been found to decrease in the order L-PAM > chlorambucil > HN2 > thioTEPA > busulfan; this is remarkable insofar as the broad-spectrum activities of these agents against rodent neoplasms appear to decrease in the same sequence in Schmidt's definitive study [analyzed by Montgomery (28)], which unfortunately antedated the advent of many of the newer agents of Table 2. The nucleophilic selectivity of cyclophosphamide metabolites may not be adequately represented by nor-HN2 and PM·CHA, but both do show high $\log(k_{s_2o_3}/k_{nbp})$ values. The lack of overlap in nucleophilic selectivity values for the mustards and the nitrosoureas could well be a factor contributing to the observation (29) of marked therapeutic synergism between these two classes.

A possible explanation for the finding that a number of alkylating agents with broad antitumor activities show high nucleophilic selectivity is that alkylation of the strongly nucleophilic N-7 position of guanine (25) is necessary for antitumor action, but that competing reactions with less nucleophilic sites in nucleic acids cause relatively more toxicity than therapeutic effect. Alkylation of nucleic acid monoanionic phosphates may occur increasingly with decreasing selectivity, resulting in formation alkyl phosphotriesters which are likely to act secondarily as alkylating centers with possibly low nucleophilic selectivity (13, 25). Of course, many factors other than nucleophilic selectivity influence patterns of nucleic acid alkylation, such as site of formation of the aziridinium ion, carrier charge, solubility characteristics, membrane transport phenomena, and DNA repair events; other linear free energy relationships, including

Brönsted and Hammett parameters, are also important determinants of alkylating agent reactivity and selectivity (11, 18, 27, 29, 30). Different linear free energy relationships may be interrelated, such as the expectation (17) that high s constant alkylating agents undergo solvolysis with reduced sensitivity to changes in the dielectric constant of the medium, represented by low Winstein m values. For general acid-catalyzed reactions, nucleophilic selectivity will theoretically be a function of the pK_a of the catalyzing acid (10).

In conclusion, it seems clear that nucleophilic selectivity has been an important yet neglected drug design variable of clinical alkylating agents. A high degree of selectivity among nucleophiles may be requisite for broad-spectrum antitumor activity for certain alkylating agent classes. Concern over the mutagenic and carcinogenic potential of an alkylating agent with poor selectivity for the stronger (more polarizable) nucleophile should be tempered by an understanding that the drug's reactivity may be dominated by basicity and steric factors (9) not considered in the present approach.

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APPENDIX

If the Swain-Scott relationship, Eq. 4, applies to all nucleophiles in the NBP reaction mixture, including solvent molecules, then the product spread characterizing an alkylating agent should be calculable to a first approximation, assuming no side reactions or secondary alkylations. Since the amount of alkyl product formed, P, is proportional to $10^{sn}[X]$, where $[X]$ and n are a nucleophile's initial concentration and n constant, then

$$P \propto 10^{sn_1}[X_1] + 10^{sn_2}[X_2] + 10^{sn_3}[X_3] \ldots \quad (10)$$

where the subscripts denote the series of nucleophiles in the reaction mixture. At a concentration of added nucleophile, say thiosulfate concentration $[a]$, which causes a 50% decrease in alkyl-NBP chromophore product(s), if NBP is the only significantly competing nucleophile, then

$$\frac{10^{sn_1}[a]}{10^{sn_2}[\text{NBP}]} = 1 \quad (11)$$

However, for poorly selective alkylating agents, weak nucleophiles will also be effective competitors for reaction, and the observed concentration of thiosulfate, $[b]$, required to decrease the NBP product by one-half will be given by Eq. 12, which ignores acetolysis as a significant reaction:

$$\frac{10^{sn_1}[b]}{10^{sn_2}[\text{NBP}] + 10^{sn_3}[\text{H}_2\text{O}] + 10^{sn_4}[\text{Tris}]} = 1 \quad (12)$$

The ratio of Eq. 11 to Eq. 12 allows calculation of the lower thiosulfate concentration $[a]$ for "corrected" $\log(k_{s_2o_3}/k_{\text{nbp}})$ determination:

$$[a] = [b] \cdot \left[\frac{10^{sn_2}[\text{NBP}]}{10^{sn_2}[\text{NBP}] + 10^{sn_3}[\text{H}_2\text{O}] + 10^{sn_4}[\text{Tris}]}\right] \quad (13)$$

Substituting n constants and nucleophile concentrations (which were essentially constant) and s^* for the electrophilicity parameter in the NBP assay gives

$$[a] = [b] \cdot \left[\frac{10^{3.5s^*}[40.6]}{10^{3.5s^*}[40.6] + 10^{0}[28696] + 10^{2.3s^*}[17.4]}\right] \quad (14)$$

Substitution of concentration $[a]$ for concentration $[b]$ into Eq. 3 at $(A_c/A_0 - 1) = 1$ (the average experimental value), and letting the correction factor = c, so that $[a] = [b]c$, leads to the conclusion that

$$\text{corrected } \log(k_{s_2o_3}/k_{\text{nbp}}) = \text{observed } \log(k_{s_2o_3}/k_{\text{nbp}}) - \log c \quad (15)$$

Correlation of corrected $\log(k_{s_2o_3}/k_{\text{nbp}})$ values and s^* values for all of the Fig. 3a alkylating agents satisfactorily reproduced the relationship predicted by Eq. 7, shown in Fig. 3b,

$$\text{corrected } \log(k_{s_2o_3}/k_{\text{nbp}}) = 2.87s^* + 0.18 \quad (16)$$
$$(r = 0.9670;\ n = 10;\ P < 0.001)$$

Since c for a given alkylating agent is independent of the nucleophile studied, s^* determinations by use of two or more nucleophile $\log(k_x/k_{\text{nbp}})$ values should not be affected by omission of consideration of minor alkylation products.

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