A Comparison of Mutagenic and Carcinogenic Activities of Aniline Mustards

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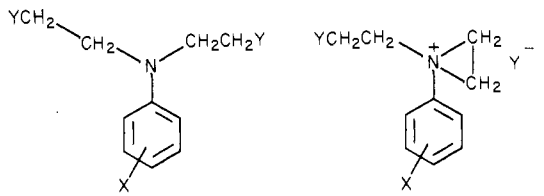
A set of 15 derivatives of aniline mustard (I) was tested to give a quantitative measure of mutagenicity in *Salmonella typhimurium* TA-1535 and TA-100 and also carcinogenicity as lung tumors in strain-A mice. The structural variation in the set was chosen to minimize collinearity between hydrophobic, electronic, and molar refractive properties. By these measures, there was *not* a direct relationship between mutagenicity and carcinogenicity; in fact, since the 4-OPh analogue ranked highest in mutagenicity and among the lowest in carcinogenicity, while the reverse was noted for the 3,5-$(NHCONH_2)_2$ analogue, an *inverse* relationship was marginally significant. S-9 activation was required in the Ames test using TA-100, and the dose-response curve, prior to toxicity, appeared biphasic.

Alkylating agents whose overall bioactivity is measured as (a) antineoplastic (prolonging life in mice challenged with P-388 lymphocytic leukemia),[1a] (b) mutagenic (Ames test with either *Salmonella typhimurium* TA-92, TA-1535, or TA-100),[2] or (c) carcinogenic (lung tumors, strain-A mouse)[3] are presumed to elicit one or more of these responses as a result of chemical interaction with nuclear DNA.[4,5] In this paper we present some measurements of the latter two types of activity with a selected set of aniline mustards (I) and our analysis of these data with emphasis



Ia Ib

on their relationship to the physical chemistry and antineoplastic activity reported and analyzed earlier.[1a] Of course, alkylating agents of the nitrogen mustard type have the potential to react with nucleophilic sites in a wide variety of biomacromolecules, and it is through quantitative structure–activity relationships (QSAR)[6] that one hopes to determine the structural parametrs to optimally direct the alkylation at the target DNA and optimize the therapeutic index.

It is well established that the active alkylating species of the aniline mustards is a cyclic aziridinium ion (Ib) which is the intermediate in hydrolysis.[7,8] Equation 1 has been formulated[1a] from the data of Ross and his colleagues on the percent hydrolysis of aniline mustards in 50% aqueous acetone at 66 °C in 30 min. It is apparent from this expression that electron-releasing groups increase the rate of hydrolysis. Since the indicator variable I_0 takes the value of 1 for ortho substitution, its positive coefficient shows that such substitution increases hydrolysis by a factor of almost 3. The indicator variable I_{Br} is assigned the value of 1 when the halogen in the aminoethyl moiety is Br and 0 for cases when it is Cl. Its positive coefficient shows that, on the average, Br is five times more rapidly hydrolyzed than Cl. The number of data points is represented by n, r is the correlation coefficient, and s is the standard deviation from the regression.

$$\log \% \text{ hyd} = -1.42\sigma + 0.45I_0 + 0.70I_{Br} + 1.21 \quad (1)$$

$$n = 42;\ r = 0.952;\ s = 0.157$$

Equation 2 correlates the antitumor (P-388 leukemia) activity of aniline mustards.[1a] C in this expression is moles per kilogram of mustard needed to give an 80% increase in life span of mice intraperitoneally inoculated with P-388 Ascites cells and then treated ip with drug. The coefficient with the σ term in eq 2 is similar to that of eq 1, suggesting that nucleophilic attack by a center in DNA is similar to nucleophilic attack by water. The indicator variable I_0 has the same meaning as in eq 1; however, its small coefficient indicates its marginal value. This is probably due to the fact that only three congeners are ortho substituted. The negative coefficient with the π term suggests that the more hydrophilic the aniline mustard, the lower the concentration needed to achieve an ILS of 80. Presumably, if a better selection of substituents with a wider range of π values were studied, an ideal π value (π_0) could be established. For the parent aniline mustard I, X = H, Y = Cl, log P = 2.90.

$$\log 1/C_{180} = -1.39\sigma - 0.34\pi + 0.30I_0 + 4.13 \quad (2)$$

$$n = 16;\ r = 0.914;\ s = 0.311$$

Experimental Section

Mutagenicity Assay. The mutagenicity assays performed at the Frederick Cancer Research Center essentially followed the procedure described by Ames et al.[9] with the following changes: 0.2 mL of the tester strain, instead of 0.1 mL; 20 mL of VBE agar, instead of 30 mL; and 75 μL of Aroclor 1254 liver fraction in 0.5 mL of S-9 mix per plate. The tester strains are grown for 14 h

(1) (a) Panthananickal, A.; Hansch, C.; Leo, A.; Quinn, F. *J. Med. Chem.* **1978**, *21*, 16. (b) Panthananickal, A.; Hansch, C.; Leo, A. *Ibid.* **1979**, *22*, 1267.
(2) Venger, B. H.; Hansch, C.; Hatheway, G. J.; Amrein, Y. U. *J. Med. Chem.* **1979**, *22*, 473.
(3) Shimkin, M. B.; Stoner, G. D. *Cancer Res.* **1975**, *21*, 1.
(4) Petzold, G. L.; Swenberg, J. A. *Cancer Res.* **1978**, *38*, 1589.
(5) Ross, W. E.; Ewig, R. A. G.; Kohn, K. W. *Cancer Res.* **1978**, *38*, 1502.
(6) Martin, Y. C. "Quantitative Drug Design"; Marcel Dekker, New York, 1978.
(7) Benn, M. H.; Kazmaier, P.; Watanatada, C. *Chem. Commun.* **1970**, 1685.
(8) Williamson, C. E.; Witten, B. *Cancer Res.* **1967**, *27*, 33.
(9) Ames, B. N.; McCann, J.; Yamasaki, E. *Mutat. Res.* **1975**, *31*, 347.

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Table I. Parameters and Activities Used to Derive Equations 4, 7, 10, and 11 for X-$C_6H_4(CH_2CH_2Cl)_2$

						log 1/B+100		log 1/Th+200		log 1/B.5		log 1/B+1	
no.	substituent	π	σ	σ^-	MR[a]	obsd	pred, eq 4	obsd	pred, eq 7	obsd	pred, eq 10	obsd	pred, eq 11
1	H	0	0	0	0.10	2.14[b]	1.285	1.94	1.645	5.06	0.02	3.65	−0.10
2	4-Cl	0.71	0.23	0.27	0.60	1.02	1.341	1.83	1.850	NC		NC	
3	4-NH_2	−1.23	−0.66	−0.15	0.54	1.40	1.348	1.28	1.083	5.55	0.01	4.07	−0.01
4	4-OCH_3	−0.02	−0.27	−0.14	0.79	1.33	1.445	1.77	1.638	4.64	0.75	3.40	0.78
5	4-$CH=C(CN)_2$	0.05	0.70	1.20	1.97	1.13	0.868	1.09[b]	1.662	4.35	0.22	2.85	0.39
6	4-CH_3	0.56	−0.17	−0.17	0.56	1.77	1.546	1.82	1.814	3.94	0.31	2.62	0.25
7	4-*n*-Bu	2.05	−0.16	−0.16	1.96	1.77	1.955	1.59	1.999	3.59	−0.01	2.22	−0.13
8	4-OC_6H_5	2.08	−0.03	−0.10	2.77	2.13	1.884	2.29	1.998	3.15	−0.22	2.10	−0.01
9	4-SO_2NH_2	−1.82	0.57	0.94	1.23	0.52	0.426	0.87	0.721	3.44	−0.30	1.66	−0.53
10	4-$NHCONH_2$	−1.30	−0.24	−0.24	1.37	1.28	1.070	1.55[b]	1.043	3.05	−0.75	1.80	−0.74
11	4-$CH=CHC_6H_5$	2.68	−0.07	0.07	3.42	1.99	2.076	2.04	1.961	NC		NC	
12	4-$CONHC_3H_7$	−0.19	0.36	0.63	1.46	1.86[b]	1.010	1.34	1.575	NC		NC	
13	4-CN	−0.57	0.66	0.89	0.63	0.57	0.720	1.04	1.417	NC		NC	
14	3,5-$(NH_2)_2$	−2.46	−0.32	−0.04	1.08	0.85	0.795	0.31	0.259	5.41	−0.34	4.12	−0.32
15	3,5-$(NHCONH_2)_2$	−2.60	−0.06	−0.06	2.74	0.31	0.596	−0.01	0.149	5.43	0.31	4.26	0.42

[a] Molar refractivity, scaled by 0.1. [b] Not used in regression.

at 37 °C in nutrient broth. Dimethyl sulfoxide is the solvent used throughout.

The test compounds were first screened using strains TA-1535, -1537, -1538, -98, and -100 to determine which strain(s) would best show mutagenicity. Dose–response curves were then done using 10 points over a 1–1000 μg dose range. Plates were incubated for 48 h at 37 °C and the revertant colonies were counted using a hand-held tally.

Pulmonary Adenoma Bioassay. The experimental details are essentially as given by Shimkin and Stoner.[3] The first step in the bioassay of the aniline mustards was to determine the maximum tolerated dose for each compound. This was accomplished by giving groups of five mice six thrice-weekly ip injections of various amounts of each compound and monitoring deaths for 2 additional weeks to detect delayed toxicity. The maximum tolerated dose was defined as the highest dose at which no deaths occurred.

In the actual bioassay, groups of 20 6-week-old strain-A mice (10 males and 10 females), obtained from the Leonell C. Strong Research Foundation, San Diego, CA, were given thrice-weekly ip injections of the maximum tolerated dose, one-fourth the maximum tolerated dose, and one-sixteenth the maximum tolerated dose. When possible, 12 injections of each dose were given, but fewer injections of the more toxic compounds were administered.

Twenty-four weeks after the first injection, the mice were sacrificed and the lungs placed in Tellyesnickzky's fluid (100 mL of 70% ethanol–5 mL of formalin–5 mL of glacial acetic acid). The lungs were then examined under a Spencer dissecting microscope [Spencer Lens Co., Buffalo, NY (10×)], and the surface adenomas were counted. A few surface nodules were examined histologically to confirm the typical morphological appearance of the adenoma.

Three of the 15 nitrogen mustards (8, 14, and 15) were obtained from the National Cancer Institute. The other 12 were synthesized at Pomona by previously described procedures.[1a,b]

Results and Discussion

In planning a set of aniline mustards for making a comparative study (QSAR) between mutagenicity and carcinogenicity, we had little information to guide us. In an initial study of the antitumor activity of aniline mustards, we found both the electronic effect of substituents and their hydrophobic effect to be important in the QSAR. Since the duration of the carcinogenicity testing was relatively long and significant expenses were involved, it was necessary at the outset to select a set of reasonable size to be tested. For this study we decided on a set size of 15, our thought being that given a good range in log P we would be able to define log P_0 (two terms π and π^2 needed) and also establish a ρ value for a σ term. Under such conditions we would have five data points per variable.

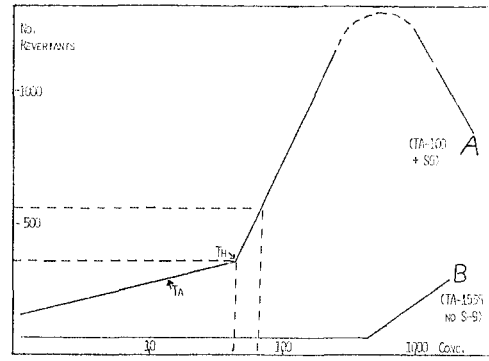

Figure 1. Typical dose–response relationship of aniline mustards in mutagenicity via base-pair substitution (Ames test): T_A = target level $\simeq$ 2 × background; T_H = threshold level.

Since all of the mustards selected had shown antitumor activity, we assumed that they were reaching DNA in mice and, hence, would be both carcinogenic and mutagenic. Unfortunately, four congeners did not show carcinogenicity under the conditions of our test which has restricted our perspective in terms of the QSAR.

Table I lists the aniline mustard derivatives, their corresponding parameters,[10] and their mutagenic and carcinogenic activities, both observed and calculated.

Mutagenicity. The results using the Ames strains TA-1535 and TA-100, which detect base-pair substitution, appear in Table II (see paragraph at the end of paper concerning supplementary material). It should be noted that activation by an S-9 microsomal fraction appeared to be absolutely required by 5 of the 15 analogues (2, 4, 5, 11, and 13), and it greatly increased the revertant rate in all of the others, except the 3,5-diamino analogue (15) which is only minimally active in any event. Previous reports have shown that other aromatic mustards—melphelan and chlornaphazin—are made more mutagenic by S-9 and that the aliphatic mustards cyclophosphamide and isophosphamide are not active without it.

Another feature worthy of note is the presence of a distinctive "threshold" concentration at which activity rises rapidly with dose; see Figure 1. For those analogues showing activity without the S-9 fraction (curve B, Figure 1), the rapid rise begins from the control revertant level.

(10) Hansch, C.; Leo, A.; Unger, S. H.; Kim, K. H.; Nikaitani, D.; Lien, E. J. *J. Med. Chem.* **1973**, *16*, 1207.
(11) Benedict, W. F.; Baker, M. W.; Haroun, L.; Choi, E.; Ames, B. N. *Cancer Res.* **1977**, *37*, 2209.

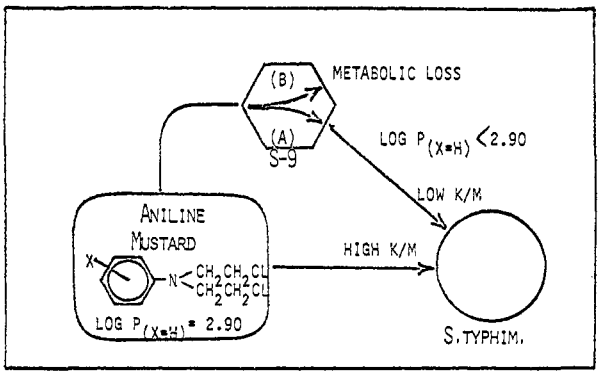

Figure 2. Proposed routes for mutation via direct alkylation (aziridinium intermediate) and activated alkylation (possible arene oxide): K/M = ratio of cell kill to cell mutation.

However, with S-9 a significant level of activity[12] is often attained before the sharp increase in slope is reached. There is a real possibility, therefore, that the introduction of microsomes results in a second pathway by which response is elicited and that the path which predominates is dependent upon concentration. In an effort to see if the two slopes in the mutagenic dose–response curve did indeed indicate separate mechanisms, we attempted to find separate QSAR for them.

For reasonable confidence in a *yes* and *no* answere to the question, "Is this compound mutagenic?", the target level used by the FCRC[12] appears justified. However, for quantitative analysis at the lowest significant activity, we preferred to use the concentration that gives a fixed revertant level above background. In other work at Pomona College we have used a lower level of the Ames TA-92 strain and have standardized on 30 drug-induced revertants per 10^8 bacteria above control. In the FCRC tests reported on here, the total count was much higher and we, therefore, chose 100 revertants above background (B+100) which produced a slightly larger spread of the data but essentially the same results as when target level was used. The results for the low-level mutagenicity are correlated by eq 3, 4, and 4a.

$$\log 1/B+100 = 0.28\ (\pm 0.12)\ \pi + 1.28\ (\pm 0.21) \quad (3)$$

$$n = 13;\ r = 0.826;\ s = 0.340;\ F_{1,11} = 23.6$$

$$\log 1/B+100 = 0.28\ (\pm 0.08)\ \pi - 0.61\ (\pm 0.35)\ \sigma + 1.28\ (\pm 0.14) \quad (4)$$

$$n = 13;\ r = 0.935;\ s = 0.224;\ F_{1,10} = 15.4$$

$$\log 1/B+100 = 0.28\ (\pm 0.14)\ \pi - 0.50\ (\pm 0.56)\ \sigma + 1.39\ (\pm 0.22) \quad (4a)$$

$$n = 15;\ r = 0.810;\ s = 0.380;\ F_{2,12} = 11.5$$

The parent compound (X = H) and the 4-CONH-Pr analogue are poorly correlated by eq 4; including them in eq 4a gives an equation with essentially the same parameters as eq 4 but with a considerably higher standard deviation. The single most important variable is π (eq 3); however, adding a term in σ yields a significantly better equation (F = 15.4). Using σ^- in place of σ gives a poorer correlation; π and σ are not collinear (r^2 = 0.0).

The negative coefficient with the electronic term in eq 4 is consistent with the activity being related to nucleophilic substitution as in eq 1 and 2, although the smaller value indicates other factors may be involved. The positive coefficient with π is important, as will be seen in eq 11 and 12, in that it can contribute to an inverse relationship between mutagenic and carcinogenic activity (eq 12).

For a measure of activity in the "after threshold" range, we chose the additional concentration required to raise the revertant level 200 counts above that at the threshold (see Figure 1). Equations 5–7 were derived from this. Two

$$\log 1/Th+200 = 0.35\ (\pm 0.13)\ \pi + 1.42\ (\pm 0.21) \quad (5)$$

$$n = 13;\ r = 0.873;\ s = 0.349;\ F_{1,11} = 35.1$$

$$\log 1/Th+200 = 0.35\ (\pm 0.14)\ \pi - 0.088\ (\pm 0.64)\ \sigma + 1.42(\pm 0.23) \quad (6)$$

$$n = 13;\ r = 0.874;\ s = 0.365;\ F_{2,10} = 16.2;\ F_{1,11} = 0.09$$

$$\log 1/Th+200 = 0.35\ (\pm 0.10)\ \pi - 0.087\ (\pm 0.059)\ \pi^2 + 1.64\ (\pm 0.22) \quad (7)$$

$$n = 13;\ r = 0.941;\ s = 0.225;\ \pi_0 = 2.02;\ F_{2,10} = 38.34;\ F_{1,11} = 10.67$$

congeners [4-CH=C(CN)$_2$ and 4-NHCONH$_2$] were omitted in formulating eq 5–7. These were included in the derivation of eq 7a which has essentially the same parameters as eq 7.

$$\log 1/Th+200 = 0.33\ (\pm 0.12)\ \pi - 0.077\ (\pm 0.07)\ \pi^2 + 1.62\ (\pm 0.25) \quad (7a)$$

$$n = 15;\ r = 0.889;\ s = 0.318;\ \pi_0 = 2.16;\ F_{2,12} = 22.5$$

A comparison of eq 5 and 6 shows that high-level mutagenicity is no longer dependent upon electronic effects but is accounted for by π alone. However, this is not greatly different from eq 4, since the σ term in eq 4 is of rather low importance.

In limited studies of compounds that do *not* require microsomal activation in the Ames test, such as phenylenediamineplatinums, nitroimidazoles, and styrene oxides,[13-15] π or log P do not appear as significant parameters. But even though it has been shown that not all microsomal oxidations are heavily dependent upon log P,[16] the role of hydrophobicity in the microsomal activation of triazines[2] and in the present case of aniline mustards appears substantial. Yet the high value for π_0 in eq 7 (2.02), coupled with the high log P of the parent compound (2.90), leads one to speculate that the hydrophobic role may not be critical in the activating reaction but in an event which begins to become rate-controlling immediately thereafter.

We have been unsuccessful in formulating a satisfactory QSAR for the aniline mustards causing mutation of either TA-1535 or TA-100 *without* activation. Five of the 15 congeners [4-OCH$_3$, 4-CN, 4-Cl, 4-CH=C(CN)$_2$ and 4-CH=CHC$_6$H$_5$] were completely inactive and the range in the remaining 10 was only a factor of 10. The most active congener in the unactivated mutation was the 4-NH$_2$ derivative.

It is interesting to note that the only two analogues to cause frame-shift mutation were those with an ethylene side chain: –CH=C(CN)$_2$ in TA-1538 and –CH=CHC$_6$H$_5$

(12) The Microbial Mutagenesis Screening Section of the Frederick Cancer Research Center uses a "target" level of revertants to distinguish between mutagens and nonmutagens. The "target" number is derived from the four plates used as the controls with each dose–response curve, i.e., the number of background revertants in two plates incorporating only the tester strain cells is added to the number of background revertants in two plates containing the cells and the solvent. The "target" level is this sum divided by two, or essentially twice background.

(13) Hansch, C.; Venger, B.; Panthananickal, A. *J. Med. Chem.* **1980**, *23*, 459.
(14) Chin, J. B.; Sheinin, D. M. K.; Routh, A. M. *Mutat. Res.* **1978**, *58*, 1.
(15) Sugiura, K.; Kumura, T.; Goto, M. *Mutat. Res.* **1978**, *58*, 159.
(16) Hansch, C. *Drug Metab. Rev.* **1973**, *1*, 1.

in TA-1538 and TA-98. The carcinogenicity of the phenylvinyl was too low to measure.

The addition of R factor plasmid (pKM 101) to the tester strain TA-1535 to give TA-100 was first studied by Ames et al.[17] and found to give increased sensitivity to polyaromatic hydrocarbons, acetylenic carbamates, and nitrofuranes. There was little difference in the overall mutation rate for this set of aniline mustards between the TA-1535 and TA-100 strains, but the data seemed a little more reliable *with* S-9 in the TA-100 and *without* S-9 in the case of the TA-1535.

A mechanism which appears to fit the available data (perhaps only one of many that would) is diagrammed in Figure 2. We may assume that upon entering the bacterial cell, the unaltered aniline mustard is much more liable to kill it than to mutate it. This high K/M ratio would be reflected in the low variance in the concentration needed to reach target level in the experiments without S-9. We postulate that inside a microsome (S-9 fraction) two reaction routes compete: reaction A, which may be epoxidation or oxidation of the amine N, would greatly lower the hydrophobicity and might also greatly lower the K/M ratio; reaction B has fewer sites, is more easily "saturated", and completely destroys the mutagenic potential of the mustards. We propose that the ratio A/B is influenced by electronic substituents and at low drug concentrations is at least partially rate limiting. At higher concentrations, the rate at which the activated mustard (now fairly hydrophilic) leaves the microsome and enters the bacterial cell becomes rate limiting. This hypothesis would explain the disappearance of the σ term and the need for a rather lipophilic substituent to counteract the hydrophilic change resulting from activation ($\pi_0 = 2.02$ in eq 7).

Carcinogenicity. Strain-A mice are the most susceptible to the development of lung tumors.[3] Although an untreated strain-A mouse will generally have developed a spontaneous lung tumor by the age of 1 year, the younger animals chosen for testing rarely have one (about 0.2 ± 0.05 in the 24-week test period). The susceptibility to chemical carcinogenesis is fetal > neonatal > adult. The difference in susceptibility between the sexes is small enough to be disregarded.[3] It should be noted that general toxicity with loss of weight can inhibit tumor formation. Examination of the dose–response curves discloses that this effect can have very little affect upon the highest activity level chosen for analysis (see eq 11 and 12).

Since they are the most costly in terms of time and material, the whole-animal studies (strain-A mouse lung tumor) placed an upper practical limit on the number of aniline mustard analogues which could be included in the comparative study. Fifteen structures were chosen on the basis of minimizing the r^2 values in a correlation matrix for hydrophobic, electronic, and molar refractivity parameters of the prospective aromatic substituents.[18] Three of these analogues (8, 14, and 15) were available from the National Cancer Institute. Eleven others had been made previously, but stocks were no longer available so further amounts were synthesized (at Pomona). One new derivative (4-CONHPr) was also synthesized to separate the hydrophobic and molar refractivity parameters.

The dose–response data observed with the selected set of aniline mustards in producing lung tumors in strain-A mice are given in Table III (supplementary material) and shown graphically in Figure 3. Four congeners (4-CH=CHC_6H_5, 4-Cl, 4-CN, and 4-CONHPr) were not active to any significant degree; in fact, at low dosages, the 4-Cl and 4-CN analogues appear to impart some marginal protective action, since the tricaprylin carrier produced a very slight increase of tumors over untreated mice (0.47 ± 0.18 vs. 0.2 ± 0.05 spontaneous tumors per mouse, untreated). As seen in Figure 3, the 3,5-$(NHCONH_2)_2$, the 3,5-$(NH_2)_2$, and the 4-NH_2 analogues produced "normal" dose–response curves, while the others gave an indication of a slower increase in activity at low dosage compared to that of high dosage. Although fewer data points are available to establish any break in the linear dose–response relationship, such as was apparent in the *Salmonella* mutagenicity discussed earlier, it appeared worthwhile to look for a different QSAR at the lowest possible activity level vs. that at a higher level. Also, in view of the established antineoplastic activity of the aniline mustards, one must consider the strong possibility that, over a 4-week dosing period, some of the tumorgenic cells produced by early doses would be destroyed by the later doses. Accordingly, it may be more informative to examine tumor response as a function of dose level rather than of total applied dose, when these are not proportional. For example, as is seen in Table III (supplementary material), toxicity considerations precluded giving more than one-half the normal course of 12 injections in the case of the 4-OCH_3 analogue.

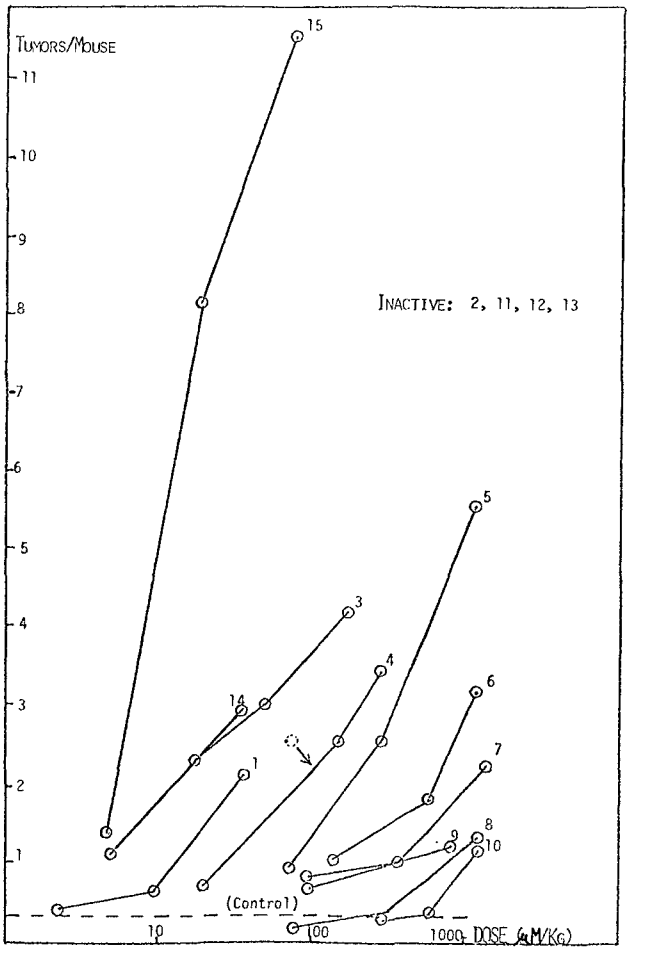

Figure 3. Dose–response relationship of aniline mustards producing lung tumors in strain-A mice.

In an effort to develop a QSAR for low-level activity, we chose the daily dose injection (moles per kilogram) needed to exceed the background produced by the tricaprylin control, that is, 0.5 tumors per mouse (B.5). Admittedly, the choice of this very low level would be difficult to justify if the possibility of a *negative* response (e.g., in the Cl and CN analogues) were discounted. For higher level activity,

(17) McCann, J.; Spingarn, N. E.; Kobori, J.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 979.
(18) See ref 6, p 238.

we used the molar concentration of *total* dose to produce one tumor above background level (B+1). The most significant relationships we could find for these activities are eq 8–10. The most significant variable is I_4 which takes

$$\log 1/\text{B.5} = -1.34\ (\pm 1.15)\ I_4 + 5.30\ (\pm 0.98) \quad (8)$$

$$n = 11;\ r = 0.657;\ s = 0.754;\ F_{1,9} = 6.85$$

$$\log 1/\text{B.5} = -1.68\ (\pm 1.0)\ I_4 + 2.40\ (\pm 2.8)\ \sigma^2 + 5.22\ (\pm 0.87) \quad (9)$$

$$n = 11;\ r = 0.788;\ s = 0.654;\ F_{1,8} = 6.55$$

$$\log 1/\text{B.5} = -1.70\ (\pm 0.83)\ I_4 + 3.30\ (\pm 2.2)\ \sigma^2 - 1.17\ (\pm 0.99)\ \sigma + 5.03\ (\pm 0.68) \quad (10)$$

$$n = 11;\ r = 0.905;\ s = 0.482;\ F_{1,7} = 10.6$$

the value of 1 for congeners having a 4-substituent and the value of 0 for all others. Having substitution in the 4 position greatly reduces (by a factor of more than 10) the carcinogenic activity of the mustards; this may be by prevention of epoxidation by microsomes.

Equations 9 and 10 have an exponential term in σ^2 which delineates an optimal σ of 0.19 and suggests an electronically dependent change in the reaction mechanism of carcinogenesis. Compounds with large positive or large negative σ values are more potent. Unfortunately, since eq 10 is based on only 11 data points, one cannot place any real confidence in it; its value lies in its suggestions of the types of derivatives which should next be prepared and tested to extend the study.

Equation 11, based on the concentration necessary to produce one tumor per mouse, is essentially the same as eq 10.

$$\log 1/\text{B+1} = -1.68\ (\pm 0.91)\ I_4 + 2.65\ (\pm 2.5)\ \sigma^2 - 1.30\ (\pm 1.09)\ \sigma + 3.75\ (\pm 0.75) \quad (11)$$

$$n = 11;\ r = 0.889;\ s = 0.530;\ \sigma_0 = 0.24;\ F_{1,7} = 8.83$$

A comparison of mutagenicity and carcinogenicity is made in eq 12 for those compounds showing both types of activity.

$$\log 1/\text{B+1 (mice)} = -0.64\ (\pm 0.92)\ \log 1/\text{Th+200 (bacteria)} + 3.82\ (\pm 1.36) \quad (12)$$

$$n = 11;\ r = 0.47;\ s = 0.90;\ F_{1,9} = 2.5$$

While it is true that many equations with *statistical* significance greater than that of the above are never examined for their possible *biochemical* import, the negative correlation found here deserves some attention, if only to assess its bearing on the current discussions concerning the dependability of all short-term mutagenic assays as predictors of human carcinogenic risk.[19] Statistically, eq 12 is significant at the 85% level as judged by the $F_{1,9}$ value of 2.5.

It has been noted previously that, within a presumed congeneric set, there is often a lack of *quantitative* correspondence between carcinogenicity and mutagenicity.[20] Two reasons proposed for the absence of the expected direct relationship are the following: (a) species and organ variability; i.e., the rat liver microsomes used in the mutagenicity studies might well activate the compounds differently than those present in skin tumorgenesis studies; (b) each carcinogen may have its own inherent specificity for the different DNA sequences involved in these mutations, and those occurring in bacteria would not bear a quantitative relationship to those initiating neoplasms in intact animals.

We believe the results reported here require us to look for another explanation. The DNA specificity proposed in reason b above *might* apply to reactive intercalating agents, but the differences within the 15 aniline mustards reported here would be minimal in this regard. Reason a is also unlikely, since the carcinogenicity tests involved ample opportunity for activation by the mouse liver before the compounds traveled from the peritoneal cavity to the lung where the tumors formed. We suggest that the mechanism outlined in Figure 2 offers a better rationale and may explain why, in the aniline mustards at least, there is *not* a *lack* of a relationship between mutagenicity and carcinogenicity but the *inverse* one seen in eq 12.

The QSAR (eq 3–7) for the mutagenic activity of the aniline mustards are reasonable first efforts and, although the number of data points per variable and the quality of fit are not high, they do point the way for new experiments. The difficulty in getting sharper equations is most likely related to the possible double mechanism of action: direct nucleophilic reaction with DNA via the aziridinium intermediate or epoxidation of the benzene ring by microsomes and then attack on the DNA.[21] Electronic demands from substituents in these two processes may differ and, if so, might cancel each other with the result that the electronic terms in eq 3–7 are not highly important. Our results do clearly show that mutagenesis through microsomal oxidation is the most important route. This came as a considerable surprise to us.

The study of the carcinogenic activity of the aniline mustards is even more complicated. No doubt the two mechanisms involved in mutagenesis can be involved in carcinogenesis. In addition, since the mustards are effective antitumor agents (eq 2), it is by no means clear to us to what extent this action inhibits their carcinogenicity.

Conclusions

One of the objectives of this study was to see if QSAR would support the hypothesis that the rate-controlling step in the case of simple alkylating agents acting as mutagens or carcinogens was the direct alkylation reaction itself. With the aniline mustards studied, this appears *not* to be the case when they are present in significant amounts. This is unexpected since the electronic effect of substituents on aniline mustards supports direct alkylation being the rate-controlling step in their action as antineoplastic agents (compare eq 1 and 2). Of course this latter cytotoxic action is not as clearly related to a reaction with DNA, for there remains a remote possibility that a key enzyme in the transformed cell is more susceptible to alkylation and that selective toxicity is exerted through protein alkylation.[22] But other evidence[4,5] led us to expect that DNA would be the target in all three cases: antineoplastic action, mutagenicity, and carcinogenicity.

The unexpected difference between the way in which aniline mustards act as antineoplastic agents and the way they act as cell-transforming agents is seen in a comparison of eq 2 with eq 7 and 10. Electron release is no longer important, either in base-pair substitution with microsomal activation or in high level carcinogenicity in strain-A mice.

(19) Maugh, T. H. *Science* **1978**, *202*, 37.
(20) Coombs, M. M.; Dixon, C.; Kissonerghis, A.-M. *Cancer Res.* **1976**, *36*, 4525.
(21) One of the reviewers suggests another possibility which would explain the altered K/M ratio as depicted in Figure 2 but perhaps not all the lowering of log P: difunctional alkylating agents are very cytotoxic while monofunctional agents are very powerful mutagens. The S-9 fraction may be producing "one-armed" mustards.
(22) Wheeler, G. P. *Cancer Res.* **1962**, *22*, 651.

These observations can be rationalized if we assume that DNA can be alkylated by aniline mustards directly through an aziridinium intermediate and also, if microsomal oxidases are present, through an arene oxide or perhaps an oxidized amine intermediate. If the latter mechanism has an opposite dependence upon the electronic character of ring substituents from the former or has no σ dependence at all and becomes the predominant route, then the absence of a σ term in eq 7 is to be expected.

If eq 7 and 10 are both expressing a cell-transformation process by an *activated* aniline mustard, then we must look for reasons why hydrophobic character dominates the former while electronic effects are vital in the latter. Somewhere in these differences lies the main reason the two cell-transforming processes are not directly related on a quantitative scale. We propose that in the Ames test with S-9 present the activated aniline mustards have little opportunity to react with anything other than the bacterial cells, while in the animal liver some "scavenger molecules" are also present to complete the detoxification process. Glutathione has been suggested as playing this role with arene oxides.[23] In the intact animal then, the most reactive of the activated mustards may never escape the liver and only those of low or intermediate reactivity are released to the portal vein where they eventually can find a susceptible tissue—the lungs in the case of strain-A mice.

Finally, we do not believe these results compromise the usefulness of the Ames test as a short-term assay to *identify* potential carcinogens. In contrast to this *qualitative* application, however, we think that attempts to draw *quantitative* comparisons of potential carcinogenicity from one type of mutagenicity measurement (in this case, base-pair substitution) are entirely unwarranted at our present level of understanding of the complex reactions involved.

Acknowledgment. The authors thank James Fornwald, Corinthia Brown, and Carol Smith of the FCRC for carrying out the Ames assays reported here. This work was supported by Grant CA-11110 from the National Cancer Institute and Contract N01-CM-6709 from the National Cancer Institute.

Supplementary Material Available: Table II, Ames test mutagenicity of aniline mustard derivatives, and Table III, strain-A mouse lung carcinogenicity of aniline mustard derivatives (19 pages). Ordering information is given on any current masthead page.

(23) Jerina, D. M.; Daley, J. W. *Science* **1974**, *185*, 573.