A Study of Comparative Chemical and Biological Activities of Alkylating Agents[1]

T. J. BARDOS, N. DATTA-GUPTA, P. HEBBORN, AND D. J. TRIGGLE

Departments of Medicinal Chemistry and Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo, Buffalo, New York

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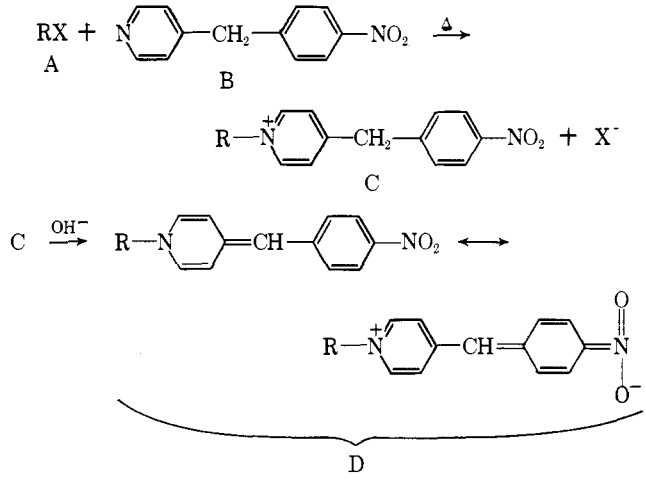
A method was developed for determining the comparative chemical alkylating activities of various types of nitrogen mustards and aziridines. A series of 10 new and 25 known compounds were studied with respect to their relative alkylating activities, rates of hydrolysis, toxicities, and tumor-inhibitory effects. Results of the chemical studies led to a new mechanistic interpretation of the SN1- and SN2-type reactions of the aromatic nitrogen mustards; clear distinction between the alternative mechanisms was possible with the aid of the newly synthesized iodo- and "branched-chain" mustards. Comparison of the relative biological activities of the latter compounds with their relative SN1- and SN2-type reactivities ("% hydrolysis" and "alkylating activity," respectively) indicated that their biologically significant *in vivo* reactions must follow an SN1 course. It is suggested that the general structure proposed for the transition state may be helpful in the design of more selective agents.

In the course of our work on the synthesis of "dual antagonists" containing an alkylating function as one of the two synergistic inhibitors incorporated into a single molecule,[2] it appeared of interest to determine the contribution of the alkylating moiety to the total biological effect of such compounds. For this purpose, we sought to develop a practical method for the comparison of the chemical reactivities of various alkylating agents with respect to a model nucleophile which would bear some resemblance to the biologically significant sites of the *in vivo* alkylation of nucleic acid purines and pyrimidines, *e.g.*, the N-7 position of guanine. This paper presents a comparative study of various types of nitrogen mustards and aziridines with respect to (1) their *in vitro* reactivities in the alkylation of the heterocyclic nitrogen of 4-(*p*-nitrobenzyl)pyridine, (2) their relative rates of hydrolysis, and (3) their biological effectiveness.

The use of 4-(*p*-nitrobenzyl)pyridine (NBP = B) as an analytical reagent for the quantitative determination of aziridines and nitrogen mustards was first proposed by Epstein, *et al.*,[3] and has been applied since by several investigators[4] for the estimation of the concentration levels of specific alkylating agents in blood and animal tissues. According to this procedure, equal volumes of various known concentration levels of a given alkylating agent are heated with a large excess of the NBP reagent for a standard period (20 min.) of time; after cooling and addition of alkali, the intensity of color development is determined spectrophotometrically by measuring the absorbances at 600-mμ wave length. By plotting these values against the concentration levels of the alkylating agent, a straight line is obtained; this is then used as the standard in the estimation of unknown concentrations of the same alkylating agent in blood or tissue extracts. The reactions leading to the formation of a colored product (D) may be represented by the following equations.



Thus, the absorbance at 600 mμ, which, according to Beer's law, is proportional to the concentration of the chromophor (D) formed upon the addition of alkali to the initial product of the alkylation reaction (C), is directly related to the extent of alkylation of B. The observed linear relationship between the alkylating agent concentration and absorbance, therefore, implies that the extent of alkylation within the given time period is proportional to the initial concentration of the alkylating agent.

In order to study the *rate* of alkylation, the above procedure was modified by varying the reaction time (heating time) at a given initial concentration of the alkylating agent. In this case, the absorbance readings were plotted against the corresponding reaction times. The points obtained throughout an initial time period (the length of which depended on the reactivity of the alkylating agent, as well as its rate of hydrolysis at the reaction temperature employed) appeared to fall on a straight line, from which they started to deviate only when the consumption of the alkylating agent in this reaction (and in the "competing" reactions, see below) resulted in a significant decrease of its initial concentration (see Figures 1 and 2).

The reaction of NBP with the alkylating agent is SN2, as indicated by its rate dependence on both the

(1) This investigation was supported by Public Health Service Research Grant No. CA-06695-02 and CA-06645-02 from the National Cancer Institute.

(2) (a) T. J. Bardos, Z. B. Papanastassiou, A. Segaloff, and J. L. Ambrus, *Nature*, **183**, 399 (1959); (b) T. J. Bardos, *Biochem. Pharmacol.*, **11**, 256 (1962); (c) T. J. Bardos and N. Datta-Gupta, Abstracts, 147th National Meeting of the American Chemical Society, Philadelphia, Pa., April 1964, p. 26M.

(3) J. Epstein, R. W. Rosenthal, and R. J. Ess, *Anal. Chem.*, **27**, 1435 (1955).

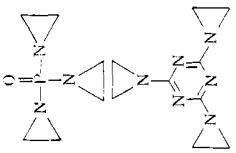
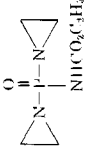
(4) (a) O. Klatt, A. C. Griffin, and J. S. Stahlin, Jr., *Proc. Soc. Exptl. Biol. Med.*, **104**, 629 (1960); (b) O. M. Friedman and E. Boger, *Anal. Chem.*, **33**, 906 (1961); (c) R. K. Ausman, G. E. Crevar, H. Hogedora, T. J. Bardos, and J. L. Ambrus, *J. Am. Med. Assoc.*, **178**, 735 (1961); (d) H. G. Petering and G. J. Van Giessen, *J. Pharm. Sci.*, **52**, 1159 (1963).

TABLE I

COMPARATIVE CHEMICAL AND BIOLOGICAL ACTIVITIES OF ALKYLATING AGENTS

No.	M	R	% hydrolysis[a]	Alkylating activity[b] $k_{80'} \times 10^3$	Alkylating activity[b] $k_{50'} \times 10^3$	Toxicity,[c] μmole/kg. Rat	Toxicity,[c] μmole/kg. Mouse	Antitumor activity,[d] ED_{90}, μmole/kg.	Antitumor activity,[d] LD_{50}/ED_{50}	Reference to source or method of synthesis
			A. Aromatic Nitrogen Mustards: M—C$_6$H$_4$—R							
1	$(ClCH_2CH_2)_2N$	H	23.2 (20°)	13	1.0	367	330	400	<1.0	*c*
2	$(ClCH_2CH_2)_2N$	COOH	2.4 (15°)	4.6	...	915	610	510	1.8	*g*
3	$(ClCH_2CH_2)_2N$	$(CH_2)_3COOH$	42[f]	41.6	2.6	92	79	7.5	12.2	*h*
4	$(ClCH_2CH_2)_2N$	CHO	<1	0.33	...	1630	2080	1150	1.4	*i*
5	$(ClCH_2CH_2)_2N$	$CONH_2$	<1[i]	0.5	...	1200	1070	1200	1.0	*j*
6	$(ClCH_2CH_2)_2N$	SO_2NH_2	2[k]	0.1	...	1130	1380	1500	<1.0	*k*
7	$(ClCH_2CH_2)_2N$	OH	60[l]	48.6	21.6	74	65	32	2.3	*m*
8	$(ClCH_2CH_2)_2N$	$OCONH\text{-}C_6H_4\text{-}COOH\text{-}p$	5.2	14	0.4	142	730	8	17.0	*m*
9	$(ClCH_2CH_2)_2N$	NH_2	100[d]	42	17.2	15	34	20	<1.0	*n*
10	$(ClCH_2CH_2)_2N$	$NHCOCH_3$	41[f]	29.6	2.0	102	98	34	3.0	*n*
11	$(ClCH_2CH_2)_2N$	$NHCOCH_2F$	19[j]	16.4	0.75	27	116	>27	<1.0	*j*
12	$(ClCH_2CH_2)_2N$	$NHCOOCH(CH_3)_2$	36 (33[m])	32	3.05	535	1630	145	3.7	*m*
13	$(ClCH_2CH_2)_2N$	$SCOCH_3$	3[n]	2.7	...	690	960	380	1.8	*o*
14	$ClCH_2CH_2NH$	H	3.2[n]	1.3	...	775	1100	>775	<1.0	*q*
15	$ClCH_2CH_2NH$	COOH	2	0	...	...	7510	...	...	*q*
16	$ClCH_2CH_2N(CH_3)$	H	11.2	4.9	0.4	3000	3300	>3000	<1.0	*r*
17	$ClCH_2CH_2N(C_2H_5)$	H	58[f]	32	5.0	1090	1770	>1090	<1.0	*s*
18	$ClCH_2CH_2N(C_2H_5)$	COOH	...	17	...	...	242	...	...	*q*
19	$ClCH(CH_3)CH_2 > N$ $ClCH_2CH_2$	SO_2NH_2	3.5	0.8	...	1870	930	1700	1.1	*q*
20	$(BrCH_2CH_2)_2N$	H	71	123	18.6	39	31	50	<1.0	*c*
21	$(BrCH_2CH_2)_2N$	COOH	16.2	46.7	...	280	250	66	4.2	*t*
22	$(BrCH_2CH_2)_2N$	$NHCOCH_3$	86	(252)	40	16	3.3	9	1.8	*q*
23	$(BrCH_2CH_2)_2N$	$SCOCH_3$	26[k]	38.9	2.5	160	315	80	2.0	*o*
24	$(BrCH_2CH_2)_2N$	SO_2NH_2	8[k]	7.5	0.4	740	558	205	3.6	*k*
25	$BrCH(CH_3)CH_2 > N$ $BrCH_2CH_2$	SO_2NH_2	37	6.1	0.67	90	140	24	3.8	*q*
26	$(ICH_2CH_2)_2N$	H	12[n]	158	20.2	140	350	82	1.7	*c*
27	$(ICH_2CH_2)_2N$	COOH	9.5	68.8	...	495	326	550	<1.0	*q*
28	$(ICH_2CH_2)_2N$	$NHCOCH_3$	55	(263)	40	31	26	50	<1.0	*q*
29	$(ICH_2CH_2)_2N$	SO_2NH_2	0	3.1	...	>1650	1250	>1650	<1.0	*q*
30	$ICH(CH_3)CH_2 > N$ ICH_2CH_2	SO_2NH_2	32.5	3.1	...	570	400	330	1.7	*q*
	Name	Formula								
			B. Nonaromatic Nitrogen Mustards							
31	Nor-HN2	$HN(CH_2CH_2Cl)_2$	3[n]	10	...	680	1000	420	1.6	*u*
32	HN2	$CH_3N(CH_2CH_2Cl)_2$	13.5[n]	(492)	80	11	16	11	1.0	*u*

C. Ethyleniminos

33	TEPA		...	3.5	...	35	155	12	2.9	*y*
34	TEM		...	6.2	...	10	10	>10	<1.0	*w*
35	AB-100		...	2.95	...	216	230	24	9	*z*

[a] Per cent hydrolysis occurring when the substance is heated in 50% aqueous acetone at 66° for 0.5 hr., determined by the method of Ross.[c] [b] $E_{600}^{1\%}/t$(min.); $k_{80}{}^{r}$, at 80°; $k_{50}{}^{r}$, at 50° (see text). [c] Approximate LI_{50} (see Experimental). [d] Against Walker carcinosarcoma 256 in rats; $EI)_{90}$ = dose required for 90% reduction of mean tumor weight; $LI)_{50}/EI)_{90}$ = "therapeutic index" (see Experimental). [e] See ref. 6a. [f] Ref. 6; values for **2** and **3** are probably for sodium salts. [g] See ref. 17. [h] Commercial preparation (Chlorambucil). [i] R. C. Elderfield, I. S. Covey, J. B. Geiduschek, W. L. Meyer, A. B. Ross, and J. H. Ross, *J. Org. Chem.*, **23**, 1749 (1958). [j] W. C. J. Ross, G. P. Warwick, and J. Roberts, *J. Chem. Soc.*, 3110 (1955). [k] L. N. Owen, A. M. Creighton, M. H. Benn, and B. J. Johnson, unpublished. [l] For literature data (probably for the hydrochloride), see ref. 6. [m] M. H. Benn, A. M. Creighton, L. N. Owen, and G. R. White, *J. Chem. Soc.*, 2365 (1961). [n] J. L. Everett and W. C. J. Ross, *ibid.*, 1972 (1949). [o] M. H. Benn, L. N. Owen, and A. M. Creighton, *ibid.*, 2800 (1958). [p] Hydrochloride; after neutralization, 9.5%. [q] See Experimental. [r] K. H. Saunders, *J. Chem. Soc.*, **121**, 2674 (1922). [s] W. R. Boon, *ibid.*, 307 (1947). [t] L. N. Owen and A. M. Creighton, unpublished. [u] Hydrochloride. [v] Hydrochloride; after neutralization, 34%. [w] Commercial preparations. [x] Hydrochloride; after neutralization, 40%. [y] H. Bestian, *Ann. Chem.*, **566**, 210 (1950). [z] Gift of Armour Pharmaceutical Co., Kankakee, Ill. (Avinar).

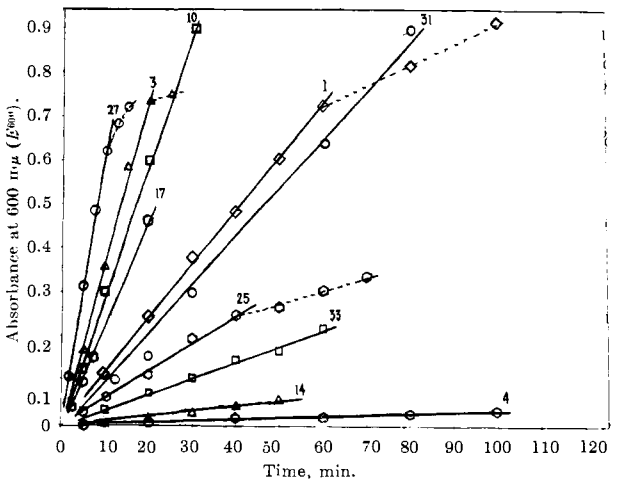


Figure 1.—Comparative reaction rates of various alkylating agents with NBP at 80°. The compounds are identified by their corresponding numbers in Table I.

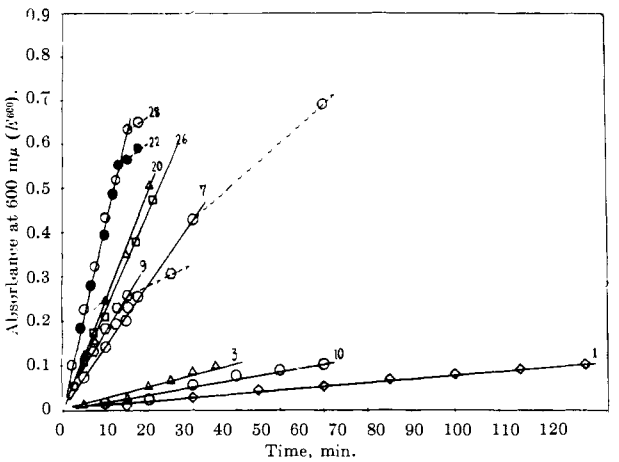


Figure 2.—Comparative reaction rates of various alkylating agents with NBP at 50°. The compounds are identified by their corresponding numbers in Table I.

alkylating agent concentration (see above) and the concentration of NBP (see Figure 3). From the integrated second-order rate equation

$$kt = \frac{1}{a - b} \ln \frac{b(a - x)}{a(b - x)}$$

where a and b are the initial concentrations of the alkylating agent and NBP, respectively, and x is the amount reacted in t time. If $b >> a$

$$kt = -\frac{1}{b} \ln \frac{a - x}{a}$$

or

$$\frac{a - x}{a} = e^{-bkt}$$

and

$$x = a(1 - e^{-bkt})$$

Expansion of the exponential term by Maclaurin's series gives

$$x = a\left[1 - 1 + bkt - \frac{(bkt)^2}{2} + \frac{(bkt)^3}{6} - \ldots\right]$$

In the initial phase of the reaction, when $x << a$ and $bkt << 1$, the quadratic and higher power terms may

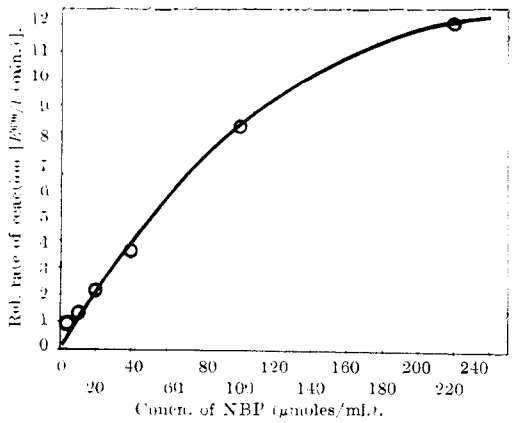


Figure 3.—Rate dependence of the alkylation reaction on the initial concentration of the nucleophile (NBP). The initial concentration of the alkylating agent (phenyl nitrogen mustard, **1**) is kept constant (0.2 μmole).

be neglected. Thus $x \sim abkt$, that is, the amount of the alkylating agent reacted with NBP *during the initial phase* of the reaction is, in first approximation, proportional to the initial concentrations of the reactants as well as to the reaction time. Since this linear relationship depends on the condition that $bkt << 1$, its validity extends through a longer time period (larger t) if k is smaller, *i.e.*, in the case of less reactive alkylating agents, or, at a lower reaction temperature. This is in agreement with the experimental results, as shown in Figures 1 and 2 [compare, *e.g.*, the absorbance *vs.* time plots for **1** at 80° (Figure 1) and at 50° (Figure 2)].

However, alkylation of NBP is not the only reaction which reduces the concentration of the alkylating agent. Taking into account the "competing" hydrolysis reaction, the corrected concentration change can be determined from the equation (for $b >> a$)

$$\frac{dx}{dt} = k(a - x)b + k_w(a - x) = (kb + k_w)(a - x)$$

in which k_w is the first-order rate constant for the hydrolysis of the alkylating agent. Integration and evaluation of the constant at zero time gives

$$\ln \frac{a}{(a - x)} = (kb + k_w)t$$

from which, by analogous treatment as above, the total amount reacted will become

$$x \sim a(kb + k_w)t$$

This relationship is based on the condition that $(kb + k_w)t << 1$. Since only by neglecting the higher power members of the corresponding Maclaurin's series can we correctly separate the terms pertaining to the alkylation and hydrolysis reaction, respectively

$$x \sim abkt + ak_wt \sim x_b + x_w$$

it follows that the expression for the alkylation reaction *per se* ($x_b \sim abkt$) is also subject to this, more stringent restriction.[5] This means that the magnitudes of both k and k_w determine how long the alkylation reaction will approximate a linear plot against time; as $(kb + k_w)t$ approaches unity, a sharp decline will occur in the rate of alkylation. That this is in agreement with experimental results is evident from comparison of the absorbance *vs.* time plots of **7** and **9**, which have nearly identical alkylating activities at 50°, but the k_w appears to be considerably higher for **9** (as evident from the "% hydrolysis" data in Table I); the latter follows a linear absorbance *vs.* time plot for 15 min., and the former for 30 min. (Figure 2).

Since the amount of alkylating agent treated with NBP (x_b) is equal to the amount of alkylation product (D) formed, which, in turn, is proportional to the absorbance at 600 mμ (E^{600})

$$x_b = [D] = \alpha E^{600} = abkt$$

from which the alkylation rate constant may be expressed as

$$k = \frac{\alpha}{ab} \frac{E^{600}}{t}$$

In comparing various alkylating agents, it may be assumed that the factor α will show little variation, since the chromophor system of D is the same in each case, and the differences in the R-groups attached to it (in most cases through an identical ethylamine chain) will have little influence on the molar absorbance at 600 mμ. By using the same initial concentrations of the reactants (a = 0.2 μmole, b = 240 μmoles), the *comparative alkylating activity* may be defined as

$$k' = \beta k = \frac{E^{600}}{t}$$

in which the new constant β includes the standard initial concentrations of the reactants. Thus, the k' values of various alkylating agents may be determined by plotting the absorbance readings *vs.* time and determining the slopes of the linear plots. In most cases, the reaction is carried out at 80° and, accordingly, k_{80}' is determined; however, for very reactive alkylating agents (high k or k_w) a lower reaction temperature has to be employed. In the latter case, the rate study is conducted at 50°, and the k_{50}' values are used for comparison.

Table I lists the k_{80}' and/or k_{50}' values for a series of 10 new and 25 known alkylating agents. For comparison, the "% hydrolysis" values are given as determined by Ross' method[6] at 60° in water–acetone; these data are related to (but, of course, not identical with) the k_w values, *i.e.*, the first-order rate constants for the hydrolysis of these compounds.[7]

It is immediately apparent from Table I that, in the aromatic nitrogen mustard series, the alkylating activity and the hydrolysis rate are *both* strikingly dependent on the basicity of the nitrogen. Electron-

(5) If further corrections are made for the reactions of the alkylating agent with the alcohol and the buffer (phthalate) ions present in the reaction mixture, the expression for the total amount reacted will be modified to

$$x \sim a(kb + k_w + k_s + k_c c)t$$

in which k_s is the first-order rate constant for the solvolytic reaction with alcohol, k_c is the second-order rate constant for the alkylation of phthalate, and c is the initial concentration of the phthalate ions ($c >> a$). The condition for the validity of this expression is that the product $(kb + k_w + k_s + k_c c)t << 1$, and this further restriction will apply also to the alkylation term, $x_b \sim abkt$.

(6) (a) W. C. J. Ross *J. Chem. Soc.*, 183 (1949); (b) *Advan. Cancer Res.*, **1**, 397 (1953); (c) *Ann. N. Y. Acad. Sci.*, **68**, 669 (1958); (d) "Biological Alkylating Agents," Butterworths, London, 1962, pp. 101-106.

(7) Ross' method was adopted because of its long-standing use for the comparison of aromatic nitrogen mustards, and because the "% hydrolysis" values determined by this method were available from the literature for some of the known compounds included in our study. It would be preferable, of course, to have comparative hydrolysis rate data for the same temperatures and solvent system as were used for the determination of alkylating activities. In subsequent studies on a few selected compounds, determination of the absolute rate constants of the alkylation reaction (k) and of the competing reactions (k_w, k_s, and k_c) in the same system will be attempted.

attracting ring substituents *para* to the nitrogen dramatically decrease, while electron-releasing substituents considerably increase the reactivities of the parent phenyl-nitrogen mustard (**1**) with respect to hydrolysis as well as nucleophilic substitution. Apparently for the same reason, "one-arm" phenyl-nitrogen mustards which have a secondary amino group (**14** and **15**) are quite inert in both types of reactions, while methyl substitution (**16**) and, particularly, ethyl substitution (**17** and **18**) on the nitrogen greatly increase their reactivities. This is in agreement with the relative basicities of the nitrogen in these compounds (compare with the pk_a values of aniline, 4.62; N-methylaniline, 4.85; and N-ethylaniline, 5.11; see also Ross[6]). In fact, the values for "% hydrolysis" and "alkylating activity" show quite good parallelism in their relative magnitudes through a whole series of mono- and difunctional aromatic nitrogen mustards which structurally differ from each other in the nature of the *para* substituent or (if monofunctional) in the N-substitution. It is evident that, in the case of the aromatic nitrogen mustards, both types of reactions, *i.e.*, the SN1-type hydrolysis and the SN2-type alkylation, are equally and decisively dependent on the magnitude of anchimeric assistance provided by the unshared electron pair of the nitrogen atom; therefore, the rate-controlling step in both reactions must involve a similar reactive intermediate (as shown below).

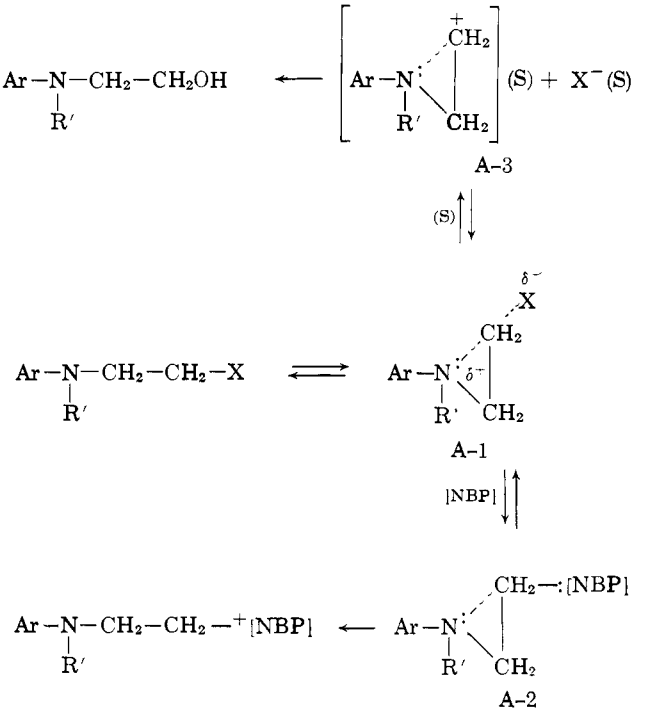
This parallelism between the alkylating activities and hydrolysis rates of aromatic nitrogen mustards breaks down in the case of alkyl substitution in the β-carbon. Compound **25**, in which one of the alkylating side chains is a 2-bromopropyl group (*i.e.*, a secondary instead of a primary halide), is considerably *more* reactive in hydrolysis and slightly *less* reactive in alkylation than its bis(2-bromoethyl) analog **24**. This divergence of relative reaction rates in the case of secondary halides gives strong support to the theory that the hydrolytic reaction follows SN1 mechanism (promoted by the polar effect of the methyl group which results in greater stability of the secondary carbonium ion intermediate) while the alkylation is a typical SN2 reaction (adversely affected by steric hindrance).

Replacement of the chlorine with bromine greatly increases the rates of both reactions due to the greater polarizability of the C–Br bond. All bromo mustards listed in Table I have much higher k' and "% hydrolysis" values than their respective chloro analogs. Replacement of the bromine with iodine appears to have a more complex effect. All iodo mustards included in our study showed considerably lower rates of hydrolysis than the corresponding bromo compounds (the same Cl < I < Br order of hydrolytic reactivity was noted by Ross[6]). On the other hand, of the same five iodo compounds, the three most reactive ones (**26**–**28**) showed either higher or equal alkylating activities than their respective bromo analogs (**20**–**22**, respectively), while the remaining two iodo compounds (**29** and **30**, members of the much less reactive sulfonamide mustard series) showed less alkylating activity than the corresponding bromo mustards (**24** and **25**, respectively). Since the C–I bond is more polarizable than the C–Br bond, the iodine is a better "leaving group" than the bromine, and iodo compounds are generally more reactive than bromo compounds in solvolysis as well as nucleophilic displacement. However, the iodide ion is also a better nucleophile than the bromide ion (the difference in nucleophilicity is much greater between I^- and Br^- than between Br^- and Cl^-),[8] and this would favor the reverse reaction, particularly if the reactive intermediate still contains the halide in a loosely bonded (or "intimate ion"[9]) form to allow internal return. The latter effect apparently predominates in the case of **29** and **30** which have the least basic nitrogen, but it is relatively less important in the alkylation reactions of **26**–**28**, in which the more basic nitrogen effectively displaces the iodine and gives rise to a reactive intermediate more closely resembling an aziridinium ion (see below). Therefore, in the latter case, the greater reactivity of the C–I bond balances or outweighs the opposing effect of the greater nucleophilicity of the iodide ion.

In order to explain the significantly lower hydrolysis rates of *all* iodo mustards, it has to be assumed that the poorer solvation of the iodide ion (in comparison to the chloride or bromide ion) provides less of the energy required for the transition state of the hydrolytic reaction, and has, therefore, an additional, comparatively adverse effect on the rate-controlling step.

On the basis of the above experimental results and considerations, the reaction mechanisms for the hydrolytic and alkylating reactions of the aromatic nitrogen mustards may be formulated as shown in Chart I.

CHART I



According to this proposed mechanism, the high-energy transition state of the hydrolytic reaction (A-3) is a solvated carbonium ion–nitrogen dipole (which has a conformation similar to an aziridine ring); collapse of this solvated ion–dipole gives the hydrolysis product. The transition state of the alkylation reaction is an

(8)(a) A. Streitweiser, Jr., "Solvolytic Displacement Reactions," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 107; (b) J. Hine, "Physical Organic Chemistry," 2nd. Ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 161.

(9) S. Winstein, E. Clippinger, A. H. Fainberg, R. Heck, and G. C. Robinson, *J. Am. Chem. Soc.*, **78**, 328 (1956).

"SN2 complex" (A-2) consisting of an unstable aziridinium ion in the state of a ring-opening attack by the nucleophilic reagent. Both reactions, however, would pass through the same reactive intermediate, A-1, which is comparable to the transition state for the formation of an aziridinium ion.

Of the two nonaromatic nitrogen mustards included in Table I, the unsubstituted nor-HN2 (**31**) appears to react *via* the same dual mechanism as proposed above for the aromatic compounds. This is indicated by the relative magnitudes of its k' and "% hydrolysis" values which fit in well with the series of aromatic nitrogen mustards. In contrast, HN2 (**30**) has a much higher alkylating activity than any of the former compounds and, at the same time, a relatively low hydrolysis rate. It is evident that this compound, having a much more basic nitrogen and, therefore, capable of forming a relatively stable aziridinium ion, reacts almost exclusively by an SN2 mechanism. This is in agreement with previously proposed mechanisms for the reactions of this agent.[10]

Compounds containing aziridine rings as alkylating functions were represented in this study by the three known antineoplastic agents, TEPA (**33**), TEM (**34**), and **35**.[11] Their comparative alkylating activities were determined by the same method and under identical conditions as used in the case of the nitrogen mustards. Their absorbance *vs.* time plots were linear through at least 60 min. (see Figure 1); their k_{80}' values (Table I) are comparable with those of the moderately reactive aromatic nitrogen mustards. The "% hydrolysis" data for these compounds would not be determined, of course, by Ross' method since they do not liberate HCl on hydrolysis; it is known, however, that these compounds rapidly hydrolyze and/or polymerize in the presence of weak acids but are relatively stable under neutral conditions.[12] The proton-catalyzed ring-opening reactions of aziridines with various nucleophiles have been characterized as following SN2 mechanisms.[13]

All compounds included in this study were tested biologically under identical assay conditions, in order to obtain toxicity and antitumor activity data that are directly comparable on a quantitative molar basis. The results are given in Table I. For the majority of the compounds, the "therapeutic" dose (ED_{90}) is nearly equal to the "toxic" dose (LD_{50}). There are three compounds in the series which have a "therapeutic index" (LD_{50}/ED_{90}) greater than 9, thus showing significant selectivity against the tumor; they are **3** (chlorambucil), **8** (the PABA-carbamate of **7**), and **35**. These compounds may owe their selectivity to favorable transport properties, to a metabolic activation,[14] or to "dual antagonism."[2] There are 7 additional compounds which show some possible indication of selectivity in this assay (LD_{50}/ED_{90} = 2–4). The monofunctional mustards, **14–18**, on the other hand, do not show any antitumor activity (which is in agreement with previous observations regarding monofunctional alkylating agents[5]).

Comparison of the biological activities of this series of compounds with their chemical reactivities leads to some interesting conclusions. First, there appears to be a much better quantitative correlation for the difunctional nitrogen mustards between the "% hydrolysis" and molar antitumor activity (ED_{90}) data (see Figure 4) than was expected by the present authors,

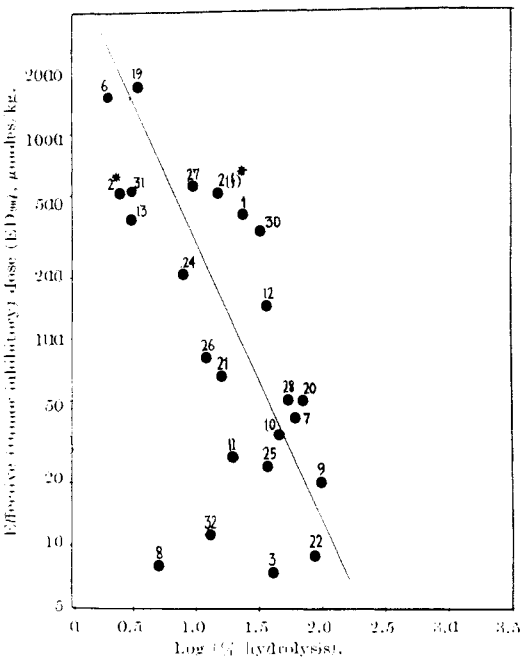


Figure 4.—Correlation of molar antitumor activities (ED_{90}) and "% hydrolysis" data for difunctional nitrogen mustards. Compounds are identified by their numbers with reference to Table I.

thus substantiating, on a more quantitative basis, the general correlation first proposed by Ross.[6] Exceptions to this rule are seen in the case of the two most selective nitrogen mustards, **3** and **8** (where *in vivo* operating factors increase the antitumor activity, see above), in the case of **11** (which, due to *in vivo* release of fluoroacetic acid, shows an increase in nonselective toxicity), and, finally, in the case of the alkyl-substituted nitrogen mustard, HN2 (**32**), which reacts by a different mechanism (see above). Second, contrary to expectation, the correlation between the "alkylating activity" and molar antitumor activity data (see Figure 5) appears to be somewhat less satisfactory. In addition to compounds **3**, **8**, and **11**, most iodo mustards (**26–29**) as well as the branched-chain bromo mustard (**25**) show substantial deviations from the general pattern of antitumor activity *vs.* chemical reactivity relationship shown by the other compounds. (In the case of **30**, the lack of "deviation" is probably due to superimposition of the effects of both iodine and β-methyl substitution, working in opposite directions.) On the other hand, HN2 (**32**) fits much better into the general pattern of nitrogen mustards. The aziridine derivatives **34** and **35** have greater biological activities than would correspond to their k_{80}' values (particularly the more selective **35**, see above).

These results indicate that, from a mechanistic point of view, the biologically significant *in vivo* reactions of

(10) (a) C. Gobmuke, J. S. Fruton, and M. Bergman, *J. Org. Chem.*, **11**, 518 (1946); (b) P. D. Bartlett, S. D. Ross, and C. G. Swain, *J. Am. Chem. Soc.*, **71**, 1415 (1949); (c) C. C. Price, *Ann. N. Y. Acad. Sci.*, **68**, 663 (1958); (d) W. C. J. Ross, ref. 6d.

(11) Z. B. Papanastassiou and T. J. Bardos, *J. Pharm. Med. Chem.*, **5**, 1000 (1962).

(12) (a) M. Beroza and A. B. Buckover, *ibid.*, **7**, 44 (1964); (b) T. J. Bardos, Z. F. Chmielewicz, and K. Navada, unpublished.

(13) G. Megnerian and L. B. Clapp, *J. Am. Chem. Soc.*, **73**, 2121 (1951).

(14) P. Hebborn and J. F. Danielli, *Biochem. Pharm.*, **1**, 19 (1958).

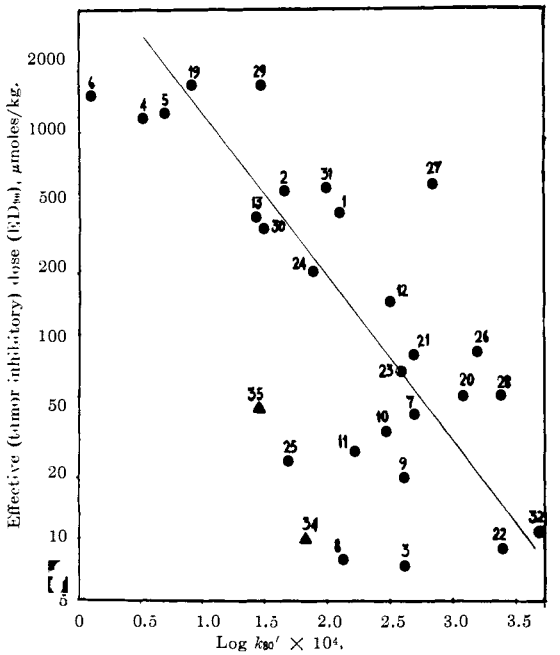


Figure 5.—Correlation of alkylating activity (k_{80}') and molar antitumor activity (ED_{90}) data for difunctional nitrogen mustards and aziridine derivatives. The compounds are identified by their numbers with reference to Table I.

the aromatic nitrogen mustards more closely parallel their SN1-type solvolysis reaction, than their SN2-type *in vitro* alkylation reaction with a heterocyclic nitrogen compound. Although the *in vitro* "alkylating activities" of these agents would be different against other nucleophiles (*e.g.*, sulfhydro compounds), the relative effects of β-methyl substitution, or, of replacement of the halogen with iodine, would be expected to remain the same; *i.e.*, as long as the reaction proceeds by an SN2 mechanism. Therefore, it seems that whatever the biologically significant reactions of these compounds may be, their rate-controlling step is the formation of a transition state more similar to a solvated carbonium ion–nitrogen dipole (A-3) than to an aziridinium ion–"SN2 complex" (A-2). It might be expected that this type of transition state would react with the surrounding water molecules to a much greater extent than with the nucleophilic groups of the biologically important cell constituents. In order to obtain selectivity, it is necessary, therefore, to design alkylating agents which would generate an A-3 type transition state only in the immediate vicinity of the desired site of action. Compound 8 seems to be an example of such an agent. Other selective compounds may be designed by steric alteration of this transition state in a manner favoring "close fit" and binding at the desired site. Preparation of new compounds based on this premise is in progress.

Experimental

New Syntheses. N-(2-Chloroethyl)aniline Hydrochloride (14).—This compound was described in the early chemical literature,[15] but the following, much simpler method was used. Aniline (100 ml., 0.93 mole) and ethylene oxide (50 ml., 1.0 mole) were dissolved in 150 ml. of absolute ethanol, and 1 g. of *p*-toluenesulfonic acid was added. Stirring was continued for 24 hr., and the product, 2-anilinoethanol, was separated by distillation; b.p. 162–163° (12 mm.), lit.[16] 157–158° (13 mm.). This compound (15 g., 0.11 mole) was allowed to react with phosphoryl chloride (12.6 ml., 0.137 mole). The yellow, viscous liquid formed was stirred for 15 min. at room temperature and for 30 min. at 50°; then 25 ml. of benzene and 250 ml. of ether were added and the mixture was cooled. A white crystalline product was obtained; m.p. 158–159°, lit.[15] 158°, yield 13 g. (56%).

***p*-[(2-Chloroethyl)amino]benzoic Acid Hydrochloride (15).**—Ethyl *p*-(2-hydroxyethyl)aminobenzoate (7 g., 0.33 mole) in chloroform (100 ml.) at 0° was treated with phosphorus pentachloride (7 g., 0.33 mole) with stirring. An immediate white precipitate occurred and the mixture was refluxed for 2 hr. and then filtered to give crude ethyl *p*-[(2-chloroethyl)amino]-benzoate. This compound was hydrolyzed to the acid by refluxing with concentrated HCl for 2 hr. Partial evaporation and chilling gave white crystals of the amino acid hydrochloride which were recrystallized from aqueous acetone to give needles (3.5 g., 34%), m.p. 215°.

Anal. Calcd. for $C_9H_{11}Cl_2NO_2$: C, 45.9; H, 4.66; Cl, 30.04. Found: C, 46.15; H, 4.68; Cl, 29.81.

***p*-[(2-Chloroethyl)ethylamino]benzoic Acid Hydrochloride (18).**—Methyl *p*-(ethylamino)benzoate (36.0 g., 0.2 mole) dissolved in 4 *N* acetic acid (250 ml.) was stirred at 0°, and ethylene oxide (9.7 g., 0.22 mole) was added. The mixture was stirred overnight at room temperature and filtered from unchanged starting material. The filtrate was neutralized and the oil was extracted with chloroform, dried (anhydrous sodium sulfate), and distilled to give **methyl *p*-[ethyl(2-hydroxyethyl)amino]benzoate** as an oil (13 g., 30%), b.p. 220° (10 mm.). The compound was apparently crude since a satisfactory elemental analysis was not obtained, and it was chlorinated and hydrolyzed according to the procedure described by Everett, *et al.*,[17] to give *p*-[(2-chloroethyl)-ethylamino]benzoic acid hydrochloride (4.4 g., 49%), m.p. 152–153°, from aqueous methanol.

Anal. Calcd. for $C_{11}H_{14}Cl_2NO_2$: C, 58.2; H, 6.21; Cl, 26.85. Found: C, 58.16; H, 6.27; Cl, 26.45.

N^4-(2-Chloroethyl)-N^4-(2-chloropropyl)sulfanilamide (19).—Propylene oxide (12.8 g., 0.22 mole) and 2-anilinoethanol (27.4 g., 0.2 mole) in benzene (50 ml.) were heated at 100° for 12 hr. and distilled to give **1-[N-(2-hydroxyethyl)anilino]-2-propanol** in near quantitative yield as a viscous oil, b.p. 210–215° (10 mm.).

Anal. Calcd. for $C_{11}H_{17}NO_2$: C, 67.7; H, 8.77. Found: C, 67.3; H, 8.6.

N-(2-Chloroethyl)-N-(2-chloropropyl)aniline was prepared from the alcohol according to the general method of Robinson and Watt.[18] It was obtained in 65% yield, as a viscous, pale yellow oil, b.p. 170–172° (10 mm.).

Anal. Calcd. for $C_{11}H_{15}Cl_2N$: C, 56.9; H, 6.51. Found: C, 57.2; H, 6.2.

A solution of the chloro compound (23.2 g., 0.1 mole) in dry carbon tetrachloride (50 ml.) was stirred at 0–5° during the dropwise addition of chlorosulfonic acid (25 ml.). The reaction mixture was heated slowly to 100° and then maintained at 120–125° for 4 hr. The mixture was poured onto ice, and the oil was extracted with five 100-ml. portions of chloroform; the chloroform extract was dried (Na_2SO_4), treated with Norit A, and stripped at 20° to give crude **N-(2-chloroethyl)-N-(2-chloropropyl)sulfanilyl chloride** as an unrecrystallizable oil. This was dissolved in dioxane (75 ml.) and added with stirring to NH_4OH (100 ml., *d* 0.880) at 10°. The mixture was then stirred vigorously at room temperature for 30 min. and poured into ice–water to give **19**, yield 14.8 g. (45%) of pure material, m.p. 109–111° (benzene).

Anal. Calcd. for $C_{11}H_{16}Cl_2N_2O_2S$: C, 42.6; H, 5.2; Cl, 22.8; N, 9.0. Found: C, 43.1; H, 5.3; Cl, 22.64; N, 8.92.

N^4-(2-Bromoethyl)-N^4-(2-bromopropyl)sulfanilamide (25).—The corresponding chloro compound (**19**) (10 g., 0.3 mole) and 20 g. of anhydrous lithium bromide in 4-methyl-2-pentanone (125 ml.) were refluxed for 6 hr. The solvent was removed and the residue was washed well with water and recrystallized from benzene to give **25** (10 g., 74%), m.p. 116.5–117.5°.

Anal. Calcd. for $C_{11}H_{16}Br_2N_2O_2S$: C, 33.1; H, 4.03; Br, 39.9; N, 7.05. Found: C, 33.4; H, 4.1; Br, 39.9; N, 7.37.

N^4-(2-Iodoethyl)-N^4-(2-iodopropyl)sulfanilamide (30).—The corresponding bromo compound (**25**) (8 g., 0.02 mole) and an anhydrous sodium iodide (20 g.) were refluxed in acetone (75 ml.)

(15) (a) J. Nemisovsky, *J. prakt. chem.*, [II] **31**, 175 (1885); (b) G. R. Clemo and W. H. Perkin, *J. Chem. Soc.*, **125**, 1804 (1924).

(16) C. L. Butler and A. G. Renfrew, *J. Am. Chem. Soc.*, **60**, 1582 (1938).

(17) L. N. Everett, J. Roberts, and W. J. C. Ross, *J. Chem. Soc.*, 2386 (1953).

(18) R. Robinson and J. S. Watt, *ibid.*, 1536 (1934).

for 10 hr. The solvent was removed and the residue was washed with water and crystallized from benzene to give **30** (7.8 g., 78%), m.p. 123.5°.

Anal. Calcd. for $C_{11}H_{16}I_2N_2O_2S$: C, 26.75; H, 3.26; I, 51.35; N, 5.67. Found: C, 27.3; H, 3.05; I, 51.2; N, 5.4.

4-[Bis(2-bromoethyl)amino]acetanilide (22) was prepared from the corresponding chloro compound (**10**) and LiBr in 67% yield, m.p. 135–137° (from benzene).

Anal. Calcd. for $C_{12}H_{16}Br_2N_2O$: C, 39.6; H, 4.4. Found: C, 40.6; H, 4.67.

4-[Bis(2-iodoethyl)amino]acetanilide (28) was prepared from the corresponding bromo compound (**22**) and NaI in 75% yield, m.p. 145–146° (benzene–ethanol, 1:1).

Anal. Calcd. for $C_{12}H_{16}I_2N_2O$: C, 31.5; H, 3.51; I, 55.4. Found: C, 31.9; H, 3.2; I, 55.1.

***p*-[Bis(2-iodoethyl)amino]benzoic acid (27)** was prepared from the corresponding bromo compound (**21**) and NaI in 82% yield, m.p. 216° (from benzene).

Anal. Calcd. for $C_{11}H_{13}I_2NO_2$: C, 29.69; H, 2.94; I, 57.03; N, 3.15. Found: C, 29.68; H, 3.02; I, 56.83; N, 3.11.

N^4,N^4-Bis(2-iodoethyl)sulfanilamide (29) was prepared from the corresponding bromo compound (**24**) and NaI in 68% yield, m.p. 163–164° (benzene).

Anal. Calcd. for $C_{10}H_{14}I_2N_2O_2S$: C, 24.9; H, 2.94; I, 52.85; N, 5.83. Found: C, 25.3; H, 2.81; I, 52.75; N, 5.8.

Alkylation Rate Studies with 4-(*p*-Nitrobenzyl)pyridine.—The alkylating agents were dissolved in ethanol at a concentration of 0.2 μmole/ml. At least four 1-ml. aliquots of each solution were pipetted into a series of test tubes. To each test tube were then added 1 ml. of ethanol, 1 ml. of NBP solution [5% w./v. 4-(*p*-nitrobenzyl)pyridine in ethanol], and 1 ml. of 0.05 *M* potassium hydrogen phthalate buffer, pH 4.2. Another series of 4–8 test tubes (to be used as respective "blanks" for each reaction time) were prepared in the same way but replacing the alkylating agent solution with 1 ml. of ethanol.

The contents of each tube were mixed thoroughly, and the tubes were placed in the water bath maintained at constant temperature (80° for k_{80}' and 50° for k_{50}' determination). Individual test tubes (including one "blank" each time) were removed from the water bath at various intervals (*e.g.*, after 5, 10, 20, and 30 min., or after 20, 40, 60, 120, and 180 min., depending on the expected reactivity of the alkylating agents) and were cooled immediately by placing them into an ice–water bath. The contents of each test tube was then brought to a total volume of 5 ml. by the addition of alcohol and mixed thoroughly.

For determining the relative concentrations of the alkylation product formed at various times, the "blank" corresponding to a given reaction time was added to 0.6 ml. of a solution of 0.1 *N* KOH in 80% (v./v.) aqueous ethanol in a "matched" colorimeter tube, mixed for 20 sec. with a Vortex mixer and then immediately used for the adjustment of the colorimeter to "100% transmission" (zero absorbance) reading. Subsequently, the contents of the reaction tubes which had been heated for the same length of time were treated similarly, one by one, with the "alkali solution" and immediately read in the colorimeter. Before reading the next series of test tubes (heated for a different time period), the colorimeter was reset to "zero absorbance" with the use of the respective "blank." All readings were taken at 600 mμ in a Bausch and Lomb (Spectronic 20) spectrophotometer. The absorbance readings were plotted against time, and the slopes of the linear plots gave directly the k' values for the alkylating agents.

Biological Test Methods. Toxicity Determinations.—Male Holtzman rats, 180–200 g., and male Swiss mice, 22–26 g., were used. The animals were fed a pelleted diet (Purina Laboratory Chow) and tap water *ad libitum*. Animal quarters were maintained at a temperature of 23.3–24.4°. Compounds, dissolved or suspended in cottonseed oil, were administered by intraperitoneal injection to groups of 3 to 6 animals/dose level. All deaths within a 24-day period were recorded and approximate LD_{50} values were estimated graphically from per cent mortality/log dose plots.

Tumor Inhibition Studies.—Walker carcinosarcoma 256 was implanted subcutaneously in the flank region of male Holtzman rats using a trochar and cannula. A compound, dissolved or suspended in cottonseed oil, was injected intraperitoneally on the day following tumor implantation. Control animals received cottonseed oil only. The rats were killed 10 days later, and the tumors were dissected out and weighed. The ratio of the mean weight of treated tumors to the mean weight of control tumors (*T/C*) was determined and plotted against log dose. The therapeutic index was obtained from the ratio LD_{50}/ED_{90}, where ED_{90} is the dose corresponding to a *T/C* ratio of 0.1.

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The Chemistry of Cephalosporins. IV. Acetoxyl Replacements with Xanthates and Dithiocarbamates

EARLE VAN HEYNINGEN AND CARTER N. BROWN

The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

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The nucleophilic displacement of the acetoxyl group of synthetic cephalosporins by alkylxanthate salts and dialkyl- and dialkylaminoalkyldithiocarbamate salts is reported. A brief discussion of the biological activity of the products is included.

As indicated in a previous report,[1] one of the several points in naturally occurring cephalosporin C amenable to variation is the 3-acetoxymethyl function. Several changes in the acyloxy group of some synthetic cephalosporins were reported. The present paper concerns another type of variation of the 3-methyl group substitution. Abraham and co-workers[2] treated cephalosporin C with a series of pyridine derivatives and displaced the acetoxyl group, forming pyridinium derivatives which possessed enhanced antibiotic activity against gram-negative organisms. We have found that other nucleophilic reagents besides pyridine will displace the acetoxyl group and, in particular, that a ready displacement occurs with xanthates and dithiocarbamates which contain bivalent sulfur of high nucleophilicity. The cephalosporin chosen for modification was sodium 7-(2-thiopheneacetamido)-cephalosporanate (I)[3] because of its high intrinsic activity and ready availability.

Nucleophilic displacement with simple xanthates and dithiocarbamates was quite general. Derivatives of II, R = O-alkyl (**1–7**, see table numeration), were

(1) E. Van Heyningen, *J. Med. Chem.*, **8**, 22 (1965).

(2) C. W. Hale, G. G. F. Newton, and E. P. Abraham, *Biochem. J.*, **79**, 403 (1961).

(3) Keflin® (cephalothin, Lilly).