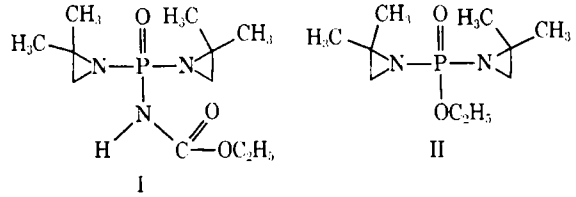
Reactions of 2,2-Dimethylaziridine-Type Alkylating Agents in Biological Systems I: Colorimetric Estimation and Stability in Physiological Media

DAVID LALKA and T. J. BARDOS▲

Abstract □ Procedures were developed for the quantitative estimation of 2,2-dimethylaziridine containing alkylating agents in blood and urine. Rapid alkalinization of the biological samples, followed by protein precipitation and extraction of the compounds at high salt concentration into acetophenone, resulted in good recoveries. The use of a nonaqueous solvent (acetophenone) and the adoption of *N*-phenylpiperazine as the proton-accepting reagent significantly improved the sensitivity and reproducibility of the colorimetric assay with 4-(*p*-nitrobenzyl)pyridine. These improvements appear to be generally applicable to the quantitative determination of aziridine-type alkylating agents. The 2,2-dimethylaziridine agents were shown to undergo extremely rapid hydrolysis under neutral or acidic conditions and were stable only in alkaline solution. The half-lives of tris(2,2-dimethyl-1-aziridinyl)phosphine oxide, bis(2,2-dimethylaziridinyl)phosphinylurethan, and ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinate in whole blood, at 37°, pH 7.38, were 7, 11, and 18 min., respectively; the corresponding half-lives in plasma were approximately four times longer.

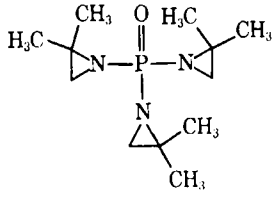
Keyphrases □ 2,2-Dimethylaziridine-type alkylating agents—stability and colorimetric estimation in blood and urine, half-lives □ Aziridine-type alkylating agents, 2,2-dimethyl analogs—stability and colorimetric estimation in blood and urine, half-lives □ pH dependence—hydrolysis rates of 2,2-dimethylaziridine-type alkylating agents □ *N*-Phenylpiperazine—used as base reagent for colorimetric estimation of 2,2-dimethylaziridine-type alkylating agents in urine and blood □ Colorimetry—analysis and stability of 2,2-dimethylaziridine-type alkylating agents in blood and urine

Bis(2,2-dimethylaziridinyl)phosphinylurethan[1] (I), one of a series of "dual antagonists" synthesized and studied in this laboratory (1–8), has been undergoing extensive clinical trials[2] as an anticancer agent (9–18). It appears that this drug potentiates the antitumor effects of X-rays clinically (14–19) as well as in animal experiments (20). In man, relatively small doses of X-rays produced short-term remissions of solid tumors when administered with Compound I (14–17). Furthermore, a recently concluded study of the treatment of bronchogenic carcinoma with this compound in combination with radiotherapy showed some encouraging long-term therapeutic effects (18, 19). These results suggest continuing interest in the clinical study of this agent. In the meantime, several other compounds containing the 2,2-dimethylaziridine moiety were synthesized and found to be effective against a spectrum of transplanted tumors in rodents (21–26). Two of these agents, ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinate (II) (23, 24) and tris(2,2-dimethyl-1-aziridinyl)phosphine oxide (III) (25), which showed particularly potent activities against leukemia L-1210 in mice, passed through the preclinical pharmacology stage (24) and are presently undergoing preliminary evaluation against solid tumors in man.



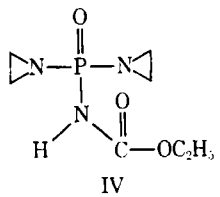
I

II

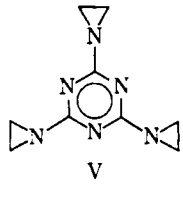


III

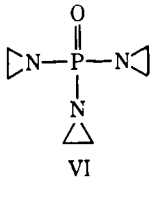
In view of the unique chemical and pharmacological properties (2, 7, 8, 22, 26) of these 2,2-dimethylaziridine derivatives as compared to their ring-C-unsubstituted aziridine analogs [*i.e.*, the conventional "ethyleniminetype" alkylating agents, bis(1-aziridinyl)phosphinylurethan (IV), triethylenemelamine (V), tris(1-aziridinyl)-phosphine oxide (VI), *etc.*] and in view of their apparently selective chemotherapeutic effects (26), it appeared worthwhile to investigate the reactions of these compounds in biological systems. Such studies may lead to a better understanding of the chemical, metabolic, and pharmacokinetic processes influencing the chemotherapeutic activities of these unusually "water-labile" alkylating agents. Therefore, the results of these studies may facilitate the design of optimally effective agents and may aid in establishing optimal conditions for their therapeutic application. The present paper describes studies to determine the *in vitro* stabilities of these agents in various aqueous and physiological media and to establish an assay methodology applicable to subsequent studies of their metabolism and distribution *in vivo*.



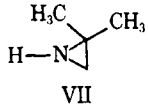
IV



V



VI



VII

[1] The following designations have been used in the literature for the agents discussed in this paper: I, AB-132, dimethylurethimine, meturedepa, NSC-51325; II, AB-163, NSC-108878; III, TEPA-132, NSC-118436; IV, AB-100, NSC-37095; V, TEM, tretamine, NSC-9706; and VI, TEPA, NSC-9717.

[2] At the Roswell Park Memorial Institute.

Table I—TLC of 2,2-Dimethylaziridine Derivatives

Compound	Solvent[a]	Absorbent	R_f Value
I	B	Alumina	0.04
II	B	Alumina	0.35
III	B	Alumina	0.24
I	B	Silica gel	0.19
II	B	Silica gel	0.24
III	B	Silica gel	0.14
VII	B	Silica gel	0.02
I	A	Silica gel	0.86
II	A	Silica gel	0.83
III	A	Silica gel	0.91
VII	A	Silica gel	0.58

[a] A, chloroform–ethanol (1:1); and B, ethyl acetate.

4-(*p*-Nitrobenzyl)pyridine (N, Scheme I) (27) is the most widely used analytical reagent for the assay of alkylating agents. Many procedures have been reported for the determination of various alkylating agents in blood and tissues using this reagent (28–35). However, the unusually rapid hydrolysis of the 2,2-dimethylaziridine derivatives in acidic or neutral aqueous media, as well as their relatively low chemical alkylating activities, necessitated the development of procedures that would provide: (*a*) rapid alkalinization followed by efficient extraction into nonaqueous media and (*b*) increased sensitivity of the colorimetric assay.

EXPERIMENTAL

Materials—Acetophenone and acetic acid were distilled before use. 4-(*p*-Nitrobenzyl)pyridine[3] and *N*-phenylpiperazine[4] were used as supplied. Compound V[5] and 2,2-dimethylaziridine[6] (VII) were purified before use. Compounds I, II, III, and VI were synthesized in this laboratory[7]. Since some of the aziridine derivatives may hydrolyze or polymerize during storage, IR and NMR spectra were taken periodically. TLC examination was also performed to check the purity and identity of the samples.

Colorimetric Determination of Alkylating Agents Using Nonaqueous Medium (Procedure A)—Reagent solutions were prepared by adding 1.0 ml. of glacial acetic acid (for I, III, VI, and VII) or 1.6 ml. of glacial acetic acid (for II and V) to 20 ml. of 25% 4-(*p*-nitrobenzyl)pyridine in acetophenone. These reagent solutions should be used within 30 min. after the addition of the acetic acid because the acetate salt precipitates upon standing longer. One milliliter of the reagent solution was added to 1.0 ml. of the sample, *i.e.*, an acetophenone solution containing the alkylating agent. The mixture was heated for 35 min. on an oil bath at 85° (55° in the case of the volatile Compound VII). To terminate the reaction, the tubes were placed into cold tap water. The color was developed by addition of 0.50 ml. of *N*-phenylpiperazine. The volume was then made up to 3.0 ml. with acetophenone. This mixture was transferred to a cell, and the absorbance at 575 nm. was measured spectrophotometrically against a blank prepared exactly as the sample except that the alkylating agent was omitted. The absorbance of the colored product is stable for at least 5 min.

Extraction of Alkylating Agents from Fresh Heparinized Blood (Procedure B)—Eight milliliters of human or dog blood was drawn into a 10-ml. syringe containing about 0.1 ml. of heparin injection USP (1000 units/ml.). The contents of the syringe were quickly emptied into a centrifuge tube containing the given amount of alkylating agent. After a few seconds of mixing[8], the centrifuge tubes were placed into an ice water bath. Then 0.5 ml. of 0.5 *M* NaOH was slowly added while vigorously shaking the tube on a mixer[8] (excessively high local concentrations of sodium hydroxide cause hemolysis). The tubes were kept in an ice water bath until they were centrifuged for the separation of the plasma. This was carried out at 1500×*g* for 10 min. at 10°. The preceding steps should be accomplished in less than 15 min. from the time the blood is drawn, and the temperature of the samples should be maintained at 0–10°.

Subsequently, 1.5-ml. aliquots of plasma were further alkalinized by the addition of 0.25 ml. of 50% NaOH. After this alkalinization, the tubes can be kept in ice water for at least 2 hr. without significant loss of activity. To each aliquot was added, in the following sequence, 1.5 ml. of 5% $ZnSO_4$, 1.5 ml. of 0.3 *N* $Ba(OH)_2$, 4.5 ml. of saturated ammonium sulfate, and 1.5 ml. of acetophenone. At least 30 sec. of vigorous agitation on a mixer[8] was applied after the addition of each reagent. This mixture was centrifuged (1500×*g*) for 35 min. at 10°, and about 1.1 ml. of an acetophenone phase was obtained. The colorimetric assay already described was then employed to determine the amount of alkylating agent in 1.0 ml. of the acetophenone phase. Contaminating traces of the aqueous phase or precipitated protein do not significantly affect the accuracy of the procedure.

Extraction of Alkylating Agents from Cellular Fraction of Blood—*Procedure C-1*—After separation of the plasma from formed elements as described for *Procedure B*, the cell fraction was kept in ice water except when otherwise stated. An aliquot of the cell fraction (1.5 ml.) was extracted with 2.0 ml. of 1.5% $NaHCO_3$ (adjusted to pH 8.4 with sodium hydroxide). The cells were resuspended by shaking on a mixer[8] and then centrifuged again. A 1.5-ml. aliquot of the supernate was treated in the same manner as was the plasma in *Procedure B*.

Procedure C-2—The cell fraction (1.5 ml.) was alkalinized with 0.2 ml. of 0.5 *M* NaOH. To this (under thorough mixing) was added, in rapid succession, 1.5 ml. of 5% $ZnSO_4$, 1.5 ml. of 0.3 *N* $Ba(OH)_2$, 0.2 ml. of 50% NaOH, 4.5 ml. of saturated ammonium sulfate, and 1.5 ml. of acetophenone. The sample was then centrifuged (1500×*g*, 10°) for 55 min. and about 1.1 ml. of an acetophenone phase was obtained. A 1.0-ml. aliquot of this phase was then treated as described in *Procedure A*.

Extraction of Alkylating Agents from Aqueous Buffers and Urine (Procedure D)—Buffer solution or urine (1.5 ml.) containing the alkylating agent was alkalinized with 0.2 ml. of 50% NaOH; then 1.5 ml. of saturated ammonium sulfate solution and 1.5 ml. of acetophenone were added. The mixture was agitated for about 30 sec. on a mixer[8] and then centrifuged (1500×*g*) for 10 min. at 10°. A 1.0-ml. aliquot of the acetophenone phase was spectrophotometrically assayed according to *Procedure A*. When the concentration of drug was too low, it was possible to extract 20 ml. of urine with 3.0 ml. of acetophenone by alkalinizing the urine with 2.0 ml. of 50% NaOH, adding 20 ml. of saturated ammonium sulfate (to salt-out drug and precipitated protein), and then adding 3.0 ml. of acetophenone. After mixing and centrifugation, about 2.5 ml. of an acetophenone phase was obtained. Duplicate 1.0-ml. aliquots

Table II—Determination of Alkylating Agents by *Procedure A*

Compound	Concentration Range Studied, mcg./ml. of Sample	Slope[a] Absorbance/ mcg./ml. of Sample	Slope[a] Absorbance/ μmoles/ml. of Sample
I	0.64–32.3	0.021	5.79
II	0.63–62.5	0.024	5.59
III	0.43–42.6	0.031	7.69
V	0.22–11.1	0.054	11.02
VI	0.13–13.1	0.038	6.61
VII	0.30–30.0	0.066	4.69

[a] The "slopes" of the Beer's law plots were calculated from quadruplicate absorbance measurements taken at five different concentrations in the range indicated, utilizing an Olivetti Programma 101 (least-squares fit through the origin). The precision and accuracy of the absorbance measurements were comparable to the values shown in Table III.

[3] 4-(*p*-Nitrobenzyl)pyridine was purchased from the Aldrich Chemical Co.

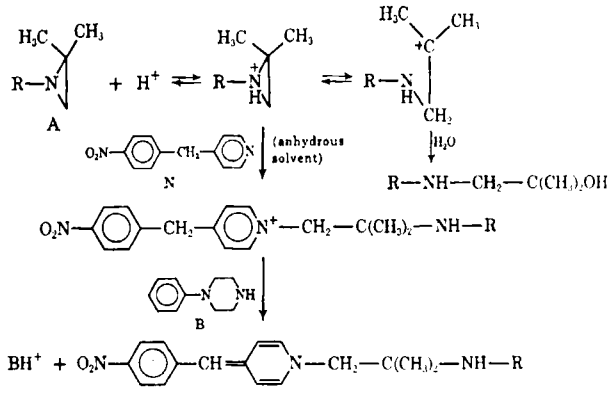
[4] *N*-Phenylpiperazine was obtained at 99% purity from the Aldrich Chemical Co. A practical grade (95% purity) was purchased from Eastman Organic Chemicals. Both products were equally satisfactory in the assay.

[5] Product of the American Cyanamid Co., purified by recrystallization from chloroform.

[6] Purchased from Fike Chemicals (through Columbia Organic) and redistilled before use.

[7] Thanks are due to Dr. Z. F. Chmielewicz and Dr. Y. Y. Hsiao for preparation of these materials.

[8] Vortex.



Scheme I—A = 2,2-dimethylaziridine-type alkylating agent (I–III, VII), N = nucleophilic reagent [4-(p-nitrobenzyl)pyridine], and B = base reagent (N-phenylpiperazine)

of this phase were then spectrophotometrically assayed according to *Procedure A*.

Chromatography (Procedure E)—TLC[9] was conducted, using two solvent systems: A, chloroform–absolute ethanol (1:1); and B, ethyl acetate. The alkylating agents were located on the chromatogram with a spray reagent. The most satisfactory reagent was a mixture of 9 ml. of 15% 4-(*p*-nitrobenzyl)pyridine in acetone and 1 ml. of glacial acetic acid (which were combined less than 5 min. before use). After spraying, the chromatogram sheets were heated to 95° in a hot air oven for 15 min. Color development was achieved by spraying with a 15% solution of *N*-phenylpiperazine in acetone (this solution is stable for about 2 days). The alkylating agents appeared as dark-blue spots on a yellow background. The R_f values are listed in Table I. Alkylating agents were extracted from plasma by adding, with vigorous shaking, in this order: 20 ml. of plasma, 3.0 ml. of 50% NaOH, 20 ml. of saturated ammonium sulfate, and 5.0 ml. of methylene chloride. Centrifugation (1500×*g*) for 35 min. at 10° yielded 1–2 ml. of a methylene chloride phase, which was then suitable for spotting.

This method permits the detection of 1–2 mcg. of alkylating agent.

RESULTS AND DISCUSSION

Assay Procedures—By using *Procedure A*, linear absorbance *versus* concentration plots (colorimetric assay standards) were obtained for 2,2-dimethylaziridine (VII), for the three agents (I–III) containing the 2,2-dimethylaziridine moiety, and for the two "conventional" alkylating agents (V and VI) containing C-unsubstituted aziridine functions. For all these compounds, good agreement with Beer's law was observed throughout the entire concentration range studied. The "slopes," given in Table II, reflect the sensitivity of the colorimetric assay. On a molar concentration basis, the slopes of the 2,2-dimethylaziridine derivatives are comparable to those of the corresponding analogous unsubstituted aziridine agents (see III *versus* VI) and are related to the number of alkylating moieties in the molecule. Thus, on a molar basis, the slopes obtained for the two bifunctional agents (I and II) are almost identical but are smaller than those obtained for the analogous trifunctional agents (III and VI) and are larger than that of the monofunctional agent (VII).

By using a nonaqueous solvent for the reaction with 4-(*p*-nitrobenzyl)pyridine, *Procedure A* eliminates the competing hydrolysis reactions for the aziridines. These reactions were particularly significant with the 2,2-dimethylaziridine derivatives (Scheme I). Rapid hydrolysis probably caused the unsatisfactory results obtained when previously published methods (using 50% aqueous buffers, usually at pH 4.5) were applied to the determination of Compound I. Acetophenone was previously employed as a solvent (36) for the colorimetric assay of alkyl halides with 4-(*p*-nitrobenzyl)pyridine; however, in the latter case, no acid was needed for the reaction to occur. In contrast, the reactions of aziridines require an appreciable

[9] On Eastman chromagram sheets (No. 6061 silica gel and No. 6062 alumina, without fluorescent indicator).

Table III—Statistical Evaluation of Results of *Procedure B* for Compounds I–III

Compound	Micrograms Added per Milliliter of Blood	Mean Absorbance[a]	Standard Deviation	Mean Error	Slope[b]
I	1.05	0.011	0.0045	−0.002	0.0127
	4.20	0.060	0.0047	+0.007	
	13.05	0.164	0.0138	−0.002	
	52.20	0.665	0.0340	+0.006	
II	0.93	0.029	0.026	+0.007	0.023
	3.70	0.089	0.017	+0.002	
	15.45	0.345	0.015	−0.017	
	61.80	1.450	0.012	−0.007	
III	0.47	0.030	0.016	+0.032	0.030
	4.04	0.150	0.009	+0.030	
	16.60	0.534	0.024	+0.011	
	66.40	2.052	0.059	−0.041	

[a] Based on four determinations. [b] Absorbance per microgram per milliliter of sample. Calculated as described in footnote of Table II.

acid concentration (see *Procedure A*), because protonation of the aziridine ring is the rate-determining step. The addition of acid to the reagent solution increases the color (absorbance) of the "blank" tubes (after alkalinization). This may be due to stabilization of the 4-(*p*-nitrobenzyl)pyridinium salt of the acid (*via* formation of a charge-transfer complex), which could favor the removal of a benzyl proton upon addition of the base. The nature of the base was a critical factor. Among a large series of bases studied, *N*-phenylpiperazine was unique in producing the maximal amount of colored product from the alkylated 4-(*p*-nitrobenzyl)pyridine in the sample tubes and, at the same time, causing very little color formation in the blanks.

Because of its overall structural similarity to 4-(*p*-nitrobenzyl)-pyridine, it is possible that the *N*-phenylpiperazine is capable of "discharging" the acid-salt complex; alternatively, *N*-phenylpiperazine is a sufficiently strong base to serve as an efficient proton

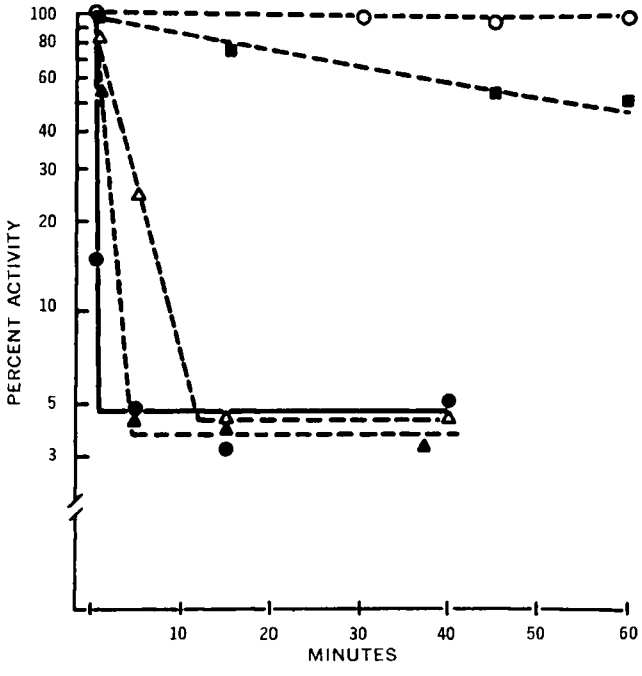


Figure 1—*Rate of hydrolysis of III in buffers of various composition (at 25°). Key: ○, pH 10.9 (0.6 M Na_2CO_3); ■, pH 7.5 (0.6 M $NaHCO_3$); △, pH 6.0 (10^{-3} M potassium hydrogen phthalate); ▲, pH 6.0 (10^{-1} M potassium hydrogen phthalate); and ●, pH 5.0 (10^{-3} M potassium hydrogen phthalate). The ordinate is the remaining alkylating activity, expressed as a percent of the t = 0 alkylating activity; t = 0 activity was calculated on the basis of an absorbance of 0.030/mcg. of III (Table III).*

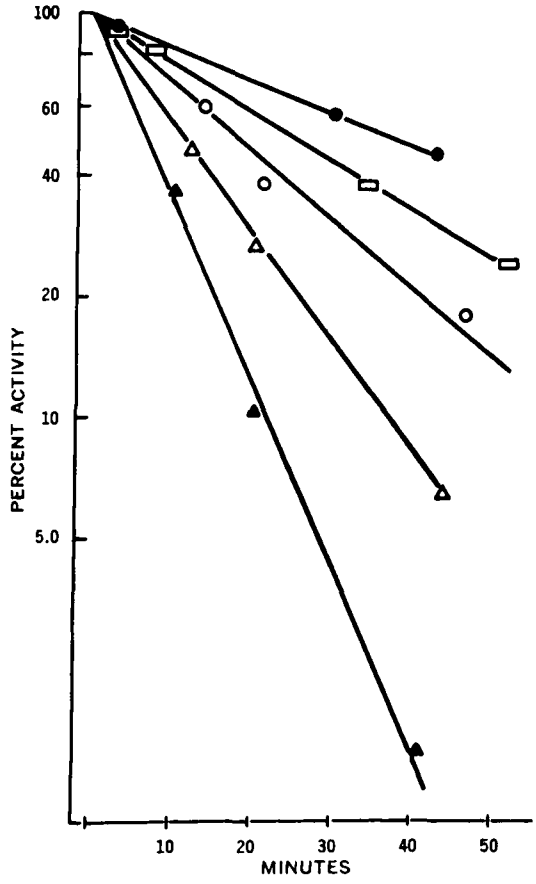


Figure 2—*Disappearance of reactivity with 4-(p-nitrobenzyl)pyridine in fresh human heparinized blood and plasma (pH 7.36). Key: ▲, III in blood at 37°; △, I in blood at 37°; ○, II in blood at 37°; □, III in plasma at 37°; and ●, III in blood at 25°.*

acceptor for a benzyl proton of the alkylated reagent but not for that of its acetate salt. The adoption of *N*-phenylpiperazine as the base reagent greatly increases the sensitivity of the colorimetric assay (Scheme I).

For the determination of 2,2-dimethylaziridine derivatives in blood samples according to *Procedure B*, it is advantageous to use another set of standard curves that already include the "recovery factor" of each agent, *i.e.*, the yield of extraction into the nonaqueous phase. Data for such curves were determined for Compounds I–III (Table III), and these data show good adherence to Beer's law. In other words, the absorbance measurements obtained in the acetophenone phase show a linear relationship to the theoretical concentrations (*i.e.*, the amount of drug per milliliter of blood added immediately before extraction) for each agent. This means that the percent recovery from blood (based on micrograms per milliliter concentration) for any given agent is constant throughout the concentration range studied, its value being dependent on the partition of the agent between the aqueous (salt) phase and the acetophenone phase (see *Procedure B*). Comparison of the data in Table III with those in Table II gives the "recovery factors": 60% for I, 95% for II, and 95% for III. The data in Table III also give some indication of the reproducibility and sensitivity of the assay procedure.

These recoveries were obtained by adding the compounds to whole blood immediately prior to separation of the plasma phase according to *Procedure B*. Virtually identical recoveries (absorbance per microgram per milliliter of biological fluid) and standard curves were obtained when the drugs were added directly to plasma. This suggests that the drugs rapidly equilibrate between the cellular fraction and plasma.

When the drugs added to the whole blood were extracted from the cell fraction, according to *Procedure C*, the percent recoveries (per milliliter of cell fraction) were somewhat lower (Table IV). This may be due to increased contact time of these reactive agents with the cells, resulting in binding and/or degradation.

Table IV—Recovery of Compounds I–III from Blood

Compound	Concentration Prepared in Whole Blood, mcg./ml.[a]	Recovery from Plasma, mcg./ml.[b,e]	Recovery from Cellular Fraction, mcg./ml.[c,e]	Recovery from Cellular Fraction, mcg./ml.[d,e]
I	78	71	49	38
II	88	76	64	39
III	68	63	46	27

[a] The alkylating agent was added to blood at 25°. [b] Results obtained utilizing *Procedure B* after the blood and alkylating agent had been incubated for 10 min. at room temperature. [c] Using *Procedure C-1*. [d] Using *Procedure C-2*. [e] The values in these columns were corrected for the 60% recovery of Compound I and the 95% recovery of Compounds II and III.

The recoveries of Compounds I–III, either from urine or from aqueous buffers, by *Procedure D* were virtually identical with the corresponding recovery factors obtained by the application of *Procedure B* to whole blood or plasma. This is significant, because it shows that the different proteins present in these biological fluids do not affect the results obtained with these assay procedures.

Stability Studies—Figure 1 shows the rates of hydrolysis of III in buffers and infusion vehicles at various pH values. The corresponding hydrolysis rates of I and II are slightly lower but show the same pH dependence. It is evident that III is stable in alkaline solution (pH 10.9) but that it loses 50% of its alkylating activity[10] in 1 hr. at pH 7.5 and in 1–3 min. at pH 6.0. At pH 5.0, about 85% of the drug is hydrolyzed in 30 sec. A 100-fold increase of the buffer concentration (at pH 6) further increases the rate of hydrolysis. Dependence of the rates of hydrolysis upon both hydrogen-ion and buffer concentrations has also been observed in the case of unsubstituted aziridine (37) and its derivatives (V and VI) (38). However, the corresponding hydrolysis rates are more than an order of magnitude lower for the latter compounds (*e.g.*, VI has a half-life of 15 hr. at pH 7.0) than for the 2,2-dimethylaziridine-containing agents. Therefore, the vehicle and rate of administration are much more critical factors in the clinical application of I–III than in the case of the conventional alkylating agents.

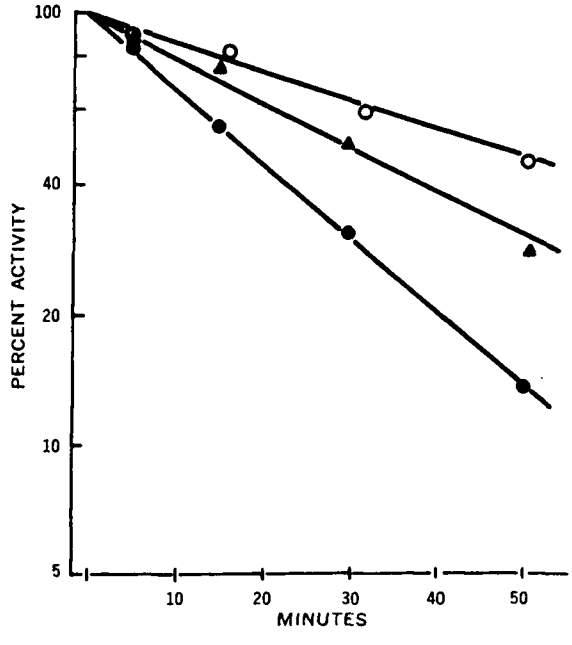


Figure 3—*Hydrolysis of Compounds I (▲), II ○, and III (●) in fresh human urine adjusted to pH 7.8 at 37°.*

[10] That is, reactivity with 4-(*p*-nitrobenzyl)pyridine, usually referred to in the literature as "NBP-reactivity."

It can be observed in Fig. 1 that about 4.5% of the zero-time alkylating activity remains after a time interval corresponding to many times the half-life of the parent compound. This suggests that the hydrolysis products contain some reactive material which is relatively stable to further hydrolysis. TLC (*Procedure E*) shows the presence of two such components, one having the same R_f values as Compound VII (Table I).

Figure 2 shows the rate of disappearance of the alkylating activities of Compounds I–III upon incubation with human blood or plasma. (Similar results were obtained with canine blood.) It is apparent that solutions of all three agents lose their alkylating activities very rapidly; the half-lives, in whole blood, at 37° are 11, 18, and 7 min. for Compounds I, II, and III, respectively. Comparison of the rates of loss of the alkylating activity for Compound III in whole blood and in plasma (half-life of 7 *versus* 26 min., respectively, at 37°) indicates that the blood cells contribute significantly to the loss of reactive material. The rate of loss was much lower at 25° than at 37° (half-life of 39 *versus* 7 min., respectively, in whole blood) (Fig. 2).

TLC analysis (*Procedure E*) of samples taken at various time points confirmed that at least 95% of the substances reacting with 4-(*p*-nitrobenzyl)pyridine were, in each case, identical with the parent compounds. Trace amounts of the two above-mentioned hydrolysis products (VII and unknown) were also detected.

The rates of hydrolysis of these agents in urine (Fig. 3) are comparable to those in blood plasma; however, substantial variations may be expected if such studies are conducted with urine samples of different compositions and pH's.

CONCLUSIONS

These studies demonstrated that the alkylating agents containing 2,2-dimethylaziridine moieties show a very rapid loss of recoverable alkylating activity when incubated with whole blood under physiological conditions. Since the half-lives of these agents in blood plasma are significantly longer, it appears that the blood cells provide effective binding sites and/or reactive nucleophilic targets for these agents. However, the possibility that some cellular enzymes (*e.g.*, phosphoramidases) may catalyze the degradation of these agents cannot be ruled out entirely.

For clinical chemotherapy, it is important to consider the extreme pH dependence of the hydrolysis rates of 2,2-dimethylaziridine-containing alkylating agents (Fig. 1). These drugs should be administered in an alkaline vehicle; otherwise, the actual dose of active drug introduced into the patient cannot be controlled.

In view of: (*a*) the relatively high and reproducible recovery of these agents from body fluids after rapid alkalinization, (*b*) the increased sensitivity of the modified colorimetric assay using nonaqueous solvent and *N*-phenylpiperazine as the base reagent, and (*c*) the relatively large doses employed clinically with the 2,2-dimethylaziridine agents, the application of these assay procedures to the study of the pharmacokinetics of these agents is feasible.

REFERENCES

(1) T. J. Bardos, Z. B. Papanastassiou, A. Segaloff, and J. L. Ambrus, *Nature*, **183,** 399(1959).

(2) T. J. Bardos, *Biochem. Pharmacol.*, **11,** 256(1962).

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

(4) T. J. Bardos, A. K. Barua, Z. F. Chmielewicz, G. E. Crevar, J. P. Dailey, S. Divald, and Z. B. Papanastassiou, *J. Pharm. Sci.*, **54,** 187(1965).

(5) T. J. Bardos, Z. F. Chmielewicz, and K. Navada, *ibid.*, **54,** 399(1965).

(6) N. Back, T. J. Bardos, Z. F. Chmielewicz, E. Achter, and A. E. Munson, *Life Sci.*, **3,** 803(1964).

(7) C. K. Navada, Z. F. Chmielewicz, and T. J. Bardos, *J. Pharm. Sci.*, **59,** 1079(1970).

(8) T. J. Bardos and J. L. Ambrus, *Int. Congr. Chemother., Proc. 3rd*, **1964,** 1036.

(9) C. A. Ross, J. E. Sokal, J. L. Ambrus, D. V. Razis, and T. J. Bardos, *Cancer Chemother. Rep.*, **3,** 263(1961).

(10) A. L. Watne, G. E. Moore, C. A. Ross, and J. L. Ambrus, *ibid.*, **16,** 421(1962).

(11) C. A. Ross, J. L. Ambrus, J. E. Sokal, H. A. Velasco, L. Stutzman, and D. V. Razis, *ibid.*, **18,** 27(1962).

(12) L. Stutzman, D. V. Razis, J. E. Sokal, J. L. Ambrus, and C. A. Ross, *ibid.*, **18,** 31(1962).

(13) B. D. Delta, C. M. Ambrus, J. L. Ambrus, and L. Stutzman, *ibid.*, **18,** 37(1962).

(14) C. A. Ross, H. A. Velasco, J. E. Sokal, J. L. Ambrus, L. Stutzman, J. Webster, and T. J. Bardos, *Proc. Amer. Ass. Cancer Res.*, **3,** 355(1962).

(15) A. L. Watne, M. A. Bozzini, F. A. Mukhtar, F. C. Marchetta, and G. E. Moore, *Cancer Chemother. Rep.*, **25,** 101(1962).

(16) L. Castro and M. M. Hreshchyshyn, *Obstet. Gynecol.*, **23,** 551(1964).

(17) H. A. Velasco, C. A. Ross, J. H. Webster, J. E. Sokal, L. Stutzman, and J. L. Ambrus, *Cancer*, **17,** 841(1964).

(18) J. H. Webster, J. Sokal, C. A. Ross, H. A. Velasco, J. L. Ambrus, and L. Stutzman, *Cancer Bull.*, **22,** 59(1970).

(19) T. J. Bardos, J. L. Ambrus, and C. M. Ambrus, *J. Surg. Oncol.*, **3,** 431(1971).

(20) W. Regelson and O. Pierucci, *Radiat. Res.*, **22,** 368(1964).

(21) Z. F. Chmielewicz, T. J. Bardos, N. Back, J. L. Ambrus, and C. M. Ambrus, *Fed. Proc.*, **26,** 686(1967).

(22) T. J. Bardos, Z. F. Chmielewicz, J. L. Ambrus, and N. Back, *Int. Congr. Chemother. 5th*, **1967,** 191.

(23) Z. F. Chmielewicz, T. J. Bardos, A. Munson, H. L. Babbitt, and J. L. Ambrus, *J. Pharm. Sci.*, **56,** 1179(1967).

(24) A. Munson, H. L. Babbitt, Z. F. Chmielewicz, T. J. Bardos, and J. L. Ambrus, *J. Surg. Oncol.*, **1,** 167(1969).

(25) A. Munson, H. L. Babbitt, A. Segaloff, Z. F. Chmielewicz, T. J. Bardos, and J. L. Ambrus, *Cancer Chemother. Rep.*, **51,** 253 (1967).

(26) T. J. Bardos, Z. F. Chmielewicz, and P. Hebborn, *Ann. N. Y. Acad. Sci.*, **163,** 1006(1969).

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

(28) O. Klatt, A. C. Griffin, and J. S. Stehlin, *Proc. Soc. Exp. Biol. Med.*, **104,** 629(1960).

(29) O. M. Friedman and E. Boger, *Anal. Chem.*, **33,** 906(1961).

(30) R. K. Ausman, G. E. Crevar, H. Hagedorn, T. J. Bardos, and J. L. Ambrus, *J. Amer. Med. Ass.*, **178,** 735(1961).

(31) H. G. Petering and G. J. Van Giessen, *J. Pharm. Sci.*, **52,** 1159(1963).

(32) R. Truhaut, E. Lelacoux, G. Brule, and C. Bohuon, *Clin. Chim. Acta*, **8,** 235(1963).

(33) Y. L. Tan and D. R. Cole, *Clin. Chem.*, **11,** 58(1965).

(34) J. C. Mattlen and L. M. McDonough, *J. Econ. Entomol.*, **60,** 1391(1967).

(35) B. T. Hill and K. R. Harrap, *Chem. Biol. Interactions*, **5,** 117(1972).

(36) E. Sawicki, D. F. Bender, T. R. Hauser, R. M. Wilson, and J. E. Mecker, *Anal. Chem.*, **35,** 1479(1963).

(37) J. G. Pomonis, R. F. Severson, P. A. Hermes, R. G. Zaylskie, and A. C. Terranova, *ibid.*, **43,** 1709(1971).

(38) M. Beroza and A. Borkovec, *J. Med. Chem.*, **7,** 44(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 15, 1973, from the *Departments of Biochemical Pharmacology and Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication March 15, 1973.

Supported by U. S. Public Health Service Research Grant RO1-CA06695 from the National Cancer Institute.

▲ To whom inquiries should be directed.