cytotoxicity (5). In attempts to develop radiosensitizers relatively less toxic than misonidazole, the results obtained upon introducing an acetyl function at the 4-position of 2-nitroimidazole have been described in this report. The acetyl function, although relatively less powerful than the nitro group with respect to the electron withdrawing capacity, has been reported to possess radiosensitizing properties (11). It was contemplated that the insertion of an acetyl function in the 2-nitroimidazole molecule may provide analogs which are comparatively less toxic than the 2,4-dinitroimidazoles. The *in vivo* acute toxicity of these agents was assessed by determining the LD_{50} in C57BL mice. Compounds V and VI were found to have an LD_{50} of 1.2 and 1.4 g/kg, respectively (Table I). These values are similar to misonidazole, which had an LD_{50} of 1.3 g/kg but are greater than the LD_{50} of 2,4-dinitroimidazoles (5). These agents are relatively less toxic than the corresponding dinitroimidazoles as expressed by the doses required for acute toxicity. The partition coefficients of these agents were determined in an octanol–phosphate buffer (pH 7.4) mixture. Compounds V and VI are more lipophilic than misonidazole (Table I), a property that would be expected to be favorable for *in vivo* diffusion into the hypoxic region of the tumors. This work has demonstrated that an additional electron affinic group could be inserted in the misonidazole molecule without significantly increasing the acute toxicity and yet enhancing the sensitizing efficiency. Thus, V may possess a therapeutic advantage over misonidazole as a radiosensitizer.

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The Hydrolysis of Spirohydantoin Mustard

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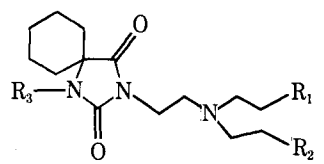
Received July 23, 1981, from the *Pharmaceutical Resources Branch, and the Laboratory of Medicinal Chemistry and Biology, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205.* Accepted for publication January 13, 1982.

Abstract □ Spirohydantoin mustard (I) is a rationally designed antitumor agent with substantial *in vivo* activity against intracranially implanted tumors in mice. However, hydrolysis of I was much faster than that of mechlorethamine hydrochloride or melphalan, two parenterally administered mustards. The hydrolysis products of I were identified by GC–MS of their silylated derivatives. The decomposition of I (at 25° in 10% dimethylacetamide at pH 4–6), as monitored by GLC was pseudo first-order. The half-life of I ranged from 20 min at pH 4.0 to 14 min at pH 6.0. Nonionic surfactants enhanced the stability of I, but this effect was diminished at lower pH, presumably due to decreased solubility of I in the micelle as more drug was protonated. Several dilute parenterally suitable solvents exhibited no marked effect on the hydrolysis of I. The drug was most stable in a 10% fat emulsion system where the time for 10% decomposition of I was 49 ± 5 min. Plots of the concentration of I *versus* time were linear indicating the disappearance was zero order in the 10% fat emulsion system.

Keyphrases □ Spirohydantoin mustard—hydrolysis, antitumor agent, mechlorethamine hydrochloride, melphalan, mice □ Hydrolysis—spirohydantoin mustard, antitumor agent, mechlorethamine hydrochloride, melphalan, mice □ Antitumor agents—hydrolysis of spirohydantoin mustard, mice

Spirohydantoin mustard (I) (NSC-172112), 3-[2-[bis(2′- chloroethyl)amino] - ethyl] - 5,5 - pentamethylenehydantoin, is a potential antitumor agent that has been designed specifically for use against central nervous system tumors. Compound I has demonstrated significant antitumor activity against L-1210 leukemia, P-388 leukemia, B-16 melanoma, and ependymoblastoma when administered in an aqueous suspension by the intraperitoneal route (1). Compound I was designed to incorporate a mustard alkylating function coupled to a substituted hydantoin carrier to provide an overall optimum partition coefficient for crossing the blood–brain barrier (1). A preliminary pharmacological study in dogs indicates that I does enter the central nervous system, although actual concentrations in cerebrospinal fluid are low (2).

As is often the case with nitrogen mustard derivatives, solution decomposition problems are evident. The aqueous stability of I and related compounds is such that octanol–water partition coefficients could not be determined (1). Preliminary chloride titration data also indicate that the decomposition of I in aqueous solution is rapid. In fact, the decomposition of I is more rapid than the hydrolysis of either of two clinically useful mustards, melphalan or mechlorethamine hydrochloride. In addition, the aqueous solubility of I is limited (<10 μg/ml) (3). Dissolution in concentrated acid with subsequent dilution is possible (1.25 mg/ml); however, the upward adjustment of pH usually results in precipitation of the drug. Likewise, I has



I $R_1 = R_2 = -Cl$; $R_3 = -H$
Ia $R_1 = R_2 = -Cl$; $R_3 = -$Trimethylsilyl or $-Si(CH_3)_3$
II $R_1 = -Cl$; $R_2 = -OH$; $R_3 = -H$
IIa $R_1 = -Cl$; $R_2 = -OSi(CH_3)_3$; $R_3 = -Si(CH_3)_3$
III $R_1 = R_2 = -OH$; $R_3 = -H$
IIIa $R_1 = R_2 = -OSi(CH_3)_3$; $R_3 = -Si(CH_3)_3$
IV $R_1 = -OH$; $R_2 = -OCOCH_3$; $R_3 = -H$
IVa $R_1 = -OSi(CH_3)_3$; $R_2 = -OCOCH_3$; $R_3 = -Si(CH_3)_3$

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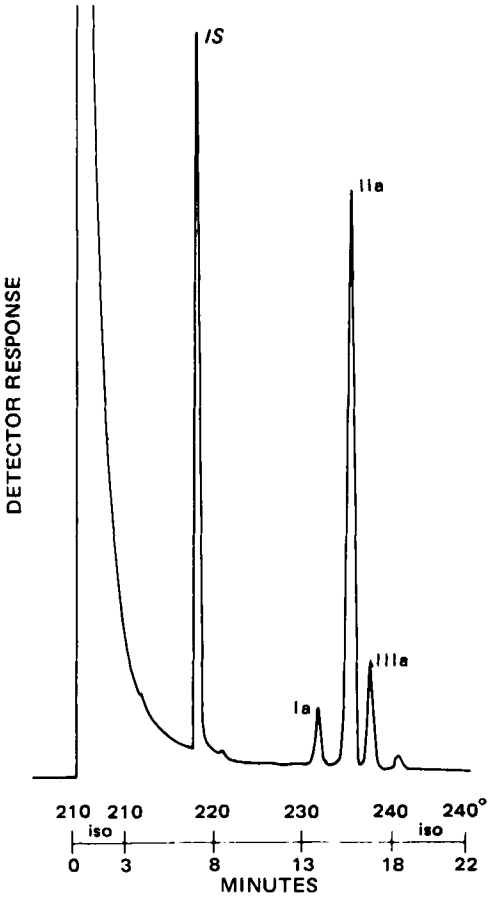


Figure 1—*Chromatogram of the trimethylsilyl derivatives of I, II, III (Ia, IIa, IIIa) and internal standard (IS) in a typical hydrolysis mixture (t = 60 min, apparent pH = 4.0).*

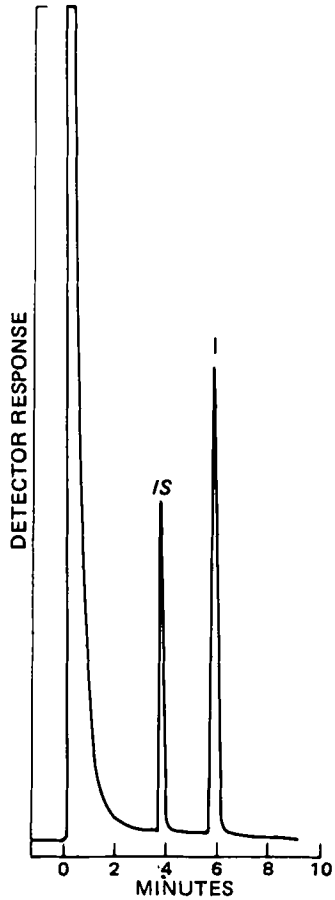


Figure 2—*Chromatogram of I and internal standard (IS) after extraction from 10% fat emulsion (1.0 mg/ml of I).*

considerable solubility in some organic solvents (*e.g.*, >300 mg/ml in dimethylacetamide); however, dilution with aqueous solutions usually causes the drug to precipitate.

The purpose of this study was to investigate the effect of pH and the presence of various excipients on the hydrolysis of I with emphasis on impeding degradation. Successful approaches would be applied to the development of a parenteral dosage form suitable for clinical trial.

EXPERIMENTAL

Materials—Dimethylacetamide[1], polysorbate 20[2], polysorbate 80[2], two polyoxyethylated castor oils[3,4], stearylcetyl alcohol ethoxylate[5], propylene glycol[6], bovine albumin[7], thymol[8], thymol blue[8], gelatin[9], spirohydantoin mustard (I)[10] (NSC-172112), mechlorethamine hydrochloride[10] (NSC-762), and melphalan (NSC-8806) were used as received. Compound III[10] was twice recrystallized from isopropanol–benzene (1:50) before use. All other chemicals were reagent grade. Citrate buffers (pH 3–6) were prepared by dilution of a 0.42 *M* stock solution of citric acid with subsequent addition of sodium hydroxide to the desired pH.

Hydrolysis Studies—Due to the limited solubility of I, studies of the hydrolysis at various pHs were conducted in 10% dimethylacetamide in citrate or ammonium acetate buffer (4.2×10^{-2} *M*), or under pH stat control. All reported pH values are in fact apparent pH values unless otherwise indicated. The mustard I was weighed, dissolved in dimethylacetamide, and then diluted with thermally equilibrated (25 ± 0.1°) water, buffer, or buffer plus excipient. Final drug concentration was 0.35 mg/ml unless otherwise indicated. All hydrolysis studies were run at 25 ± 0.1°.

Chloride Titration—The concentration of chloride ion was determined by the coulometric–amperometric titration[11] of aliquots of the hydrolysis mixture. A 0.5- or 1.0-ml aliquot was removed and diluted with 3.0 ml of 0.1 *N* nitric acid–10% acetic acid reagent. When a 0.5-ml aliquot was used, 0.5 ml of distilled water was added to keep the titration volume consistent at 4.0 ml. Four drops of a gelatin reagent containing 6.0 mg/ml of gelatin, 0.1 mg/ml of thymol blue, and 0.1 mg/ml of thymol were added prior to each titration. The titration was performed only if a red color, indicating that the mixture was sufficiently acidic (pH ~ 1.2), was present. The titrator was calibrated at the low titration rate using standard sodium chloride solutions. Blanks and standards were prepared to be of identical composition to the hydrolysis solution in terms of the buffers and excipients present.

Disappearance of I by GLC—Preliminary studies at pH 4.0 indicated that the efficient and simultaneous extraction of I, II, and III from sodium citrate or sodium acetate buffers was impractical, presumably because of the low pH. Extraction of I, II, and III could be avoided by using an ammonium acetate buffer which could be removed by freeze-drying the hydrolysis mixture. However, in this case, small peaks corresponding to acetate esters of III were detected within 90 min. Therefore, a bufferless system, which also required no extractive cleanup, was employed by using a titrator[12] operating in the pH stat mode. Sodium hydroxide (0.05 *M*) was used as the titrant. This system was capable of maintaining the pH of the solution at ±0.1 pH units during the course of the experiment.

In these hydrolysis studies a 2-ml aliquot of the hydrolysis mixture initially containing I at a concentration of 0.15 mg/ml and internal standard (phenytoin) was removed at appropriate times and immediately

[1] Burdick and Jackson Co., Muskegon, Mich.
[2] Tween 20, Tween 80, Sigma Chemical Co., St. Louis, Mo.
[3] Emulphor EL-719, GAF Corp., New York, N.Y.
[4] Cremophor EL, Sandoz, Basel, Switzerland.
[5] Siponic E-7, Alcolac Inc., Baltimore, Md.
[6] Matheson, Coleman and Bell, East Rutherford, N.J.
[7] Sigma Chemical Co., St. Louis, Mo.
[8] Fisher Scientific Co., Fair Lawn, N.J.
[9] Knox Gelatin, Inc. Johnstown, N.Y.
[10] Provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

[11] Buchler-Cotlove Chloridometer, Buchler Instruments Inc., Fort Lee, N.J.
[12] Radiometer Type TTTld Titrator, Radiometer, Copenhagen, Denmark.

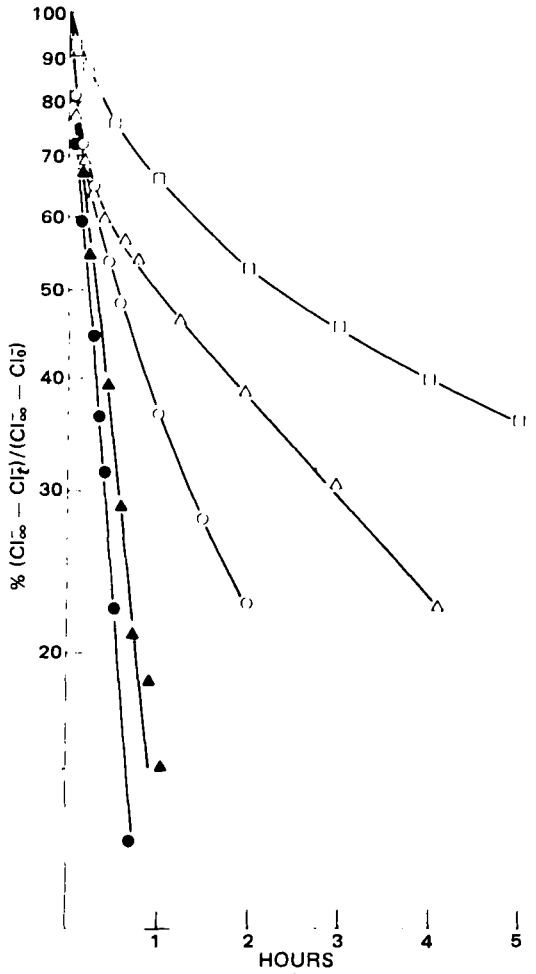


Figure 3—*The effect of pH on the hydrolysis of I (0.35 mg/ml) as determined by chloride titration. All pHs (apparent) are with sodium citrate buffer (4.2×10^{-2} M). Key: (□) pH 3.0; (△) pH 3.5; (○) pH 4.0; (▲) pH 5.0; (●) pH 6.0.*

frozen in glass vials (37.4 ml) prechilled on dry ice. Appropriate standards of I and III plus internal standard were similarly frozen. All samples were then placed on a prechilled shelf and freeze-dried[13]. The freeze-dried samples and standards were derivatized by reaction with 0.30 ml of a mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide[14]–acetonitrile (1:2) at room temperature for a minimum of 30 min. Shaking and sonication were employed to effect solution and reaction of the freeze-dried samples. Aliquots (4.0 μl) of the trimethylsilylation reaction mixture were analyzed by GLC[15] on a 1.83-m × 2-mm i.d. glass column packed with 3% SE-30 100/120 mesh Gas-Chrom Q[14] and installed for on-column injection. The gas chromatograph was temperature programmed from 210 to 240° at 2°/min after an initial delay of 3 min. Other operating parameters were: helium carrier gas flow, 30 ml/min; hydrogen, 30 ml/min; air, 300 ml/min; injector port, 255°; and flame ionization detector (FID), 260°. Samples were analyzed interspersed with standards so that standard curves of the peak area ratios of both I and III to internal standard were prepared for each hydrolysis experiment. The direct quantitation of I and III allowed II to be calculated by difference as well as a calculation of chloride ion so that a direct comparison with the coulometric–amperometric titrations could be made. A typical chromatogram is presented in Fig. 1.

GC–MS Analysis—Representative hydrolysis mixtures were also analyzed by GC–MS[16] under data system[17] control to identify the hydrolysis products. GC analysis parameters were identical to those used above, while the mass spectrometer operating conditions were: transfer line and jet separator, 255°; ion source, 260°; electron energy, 75 eV; ionizing current, 250 μamps; accelerating voltage, 1.6 kV; and scan speed, 2 sec/decade.

[13] Virtis Model 10-146 MR-BA Freeze-Mobile, The Virtis Company, Gardiner, N.Y.
[14] Applied Science Laboratories, Inc., State College, Pa.
[15] Model 2740, Varian Aerograph, Walnut Creek, Calif.
[16] DuPont 21-492B, Dupont Instruments, Monrovia, Calif.
[17] VG 2040 Data System, VG Data Systems Limited, Altrincham, England.

Table I—Comparison of Percent Total Chloride Liberated from I after Hydrolysis at pH 4.0 (apparent) in 0.042 *M* Ammonium Acetate Calculated from GLC and by Titration

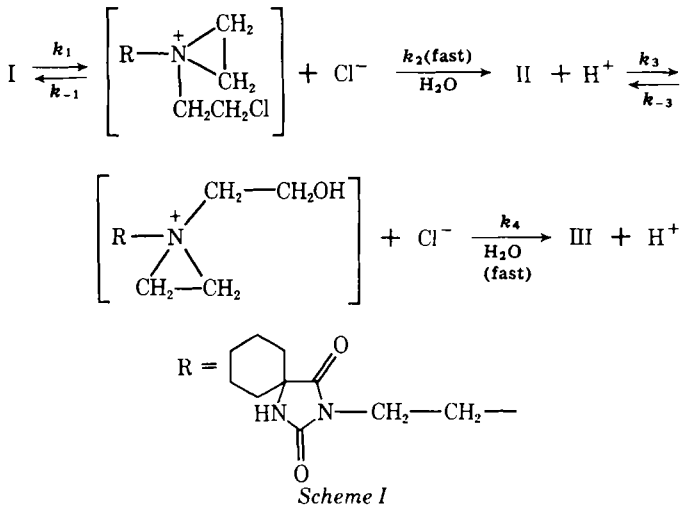
Minutes	Percent Total Chloride Liberated: Calculated from GLC Data	Percent Total Chloride Liberated: Determined by Direct Titration
5	12.3	10.3
10	16.2	16.6
20	31.7	29.8
30	39.3	38.2
60	52.1	53.0

Stability in 10% Fat Emulsion by GLC—The disappearance of I in 10% fat emulsion[18] was monitored by GLC[19]. A measured volume of 10% fat emulsion (49.4 ml) was placed in a 50-ml flint serum vial which was closed with a butyl rubber stopper and an aluminum seal. The vial was secured to a mechanical mixer with tape. The mixer was started, and a solution of I in dimethylacetamide (50 mg in 0.6 ml) was injected directly into the emulsion using a 1-ml glass syringe equipped with an 8.89-cm, 22-gauge needle. The solution of I was injected at ~1 ml/min while the vigorous mixing continued. Duplicate 2-ml aliquots were drawn at designated times, placed in glass screw-capped vials (149.6 ml each) and were immediately frozen in a dry ice–acetone bath. A series of standards corresponding to 0.2, 0.5, 0.7, 1.0, and 1.5 mg/ml of I were prepared by adding appropriate amounts of I in ethyl acetate to the emulsion. Samples were immediately frozen. All samples and standards were freeze-dried in a shelf-type unit[13]. The freeze-dried residue was extracted with 5.0 ml of ethyl acetate containing 40 mg/ml tetraphenylethylene as an internal standard. Aliquots (2 μl) of the samples and standards were analyzed by GLC on a 1.83-m × 2-mm i.d. glass column packed with 3% OV-17 on 80/100 mesh Chromosorb WHP[20] and installed for on-column injection. The gas chromatograph was operated isothermally at 230°. Other operating parameters were: helium carrier gas flow, 22 ml/min; hydrogen, 28 ml/min; air, 350 ml/min; injector port, 240°, and FID, 250°. Samples were analyzed interspersed with standards. Quantitation was accomplished by an internal standard method using the ratio of peak heights of I to tetraphenylethylene. A typical chromatogram is seen in Fig. 2.

Antitumor Activity—The antitumor activity of I in an experimental formulation (5% dimethylacetamide in 10% fat emulsion[18]) was evaluated against a suspension of bulk I in the P-388 tumor. Studies were conducted according to standard National Cancer Institute protocols (4). The tumor inoculum was injected intraperitoneally. Drug treatment began the following day and continued for a total of 9 daily intraperitoneal injections.

RESULTS AND DISCUSSION

Chloride titrations of the bulk drug I used as received indicated no free chloride was present. Hydrolysis mixtures that were allowed to proceed



Scheme I

[18] Intralipid 10%, Cutter Laboratories, Inc., Berkeley, Calif.
[19] Model 5754, Hewlett-Packard, Avondale, Pa.
[20] Supelco, Inc., Bellefonte, Pa.

Table II—GC-MS Characteristics of Silylated Derivatives of I

Compound	Ia	IIa	IIIa	IVa
Retention Index (SE-30, 220°)	2490	2543	2578	2629
Mass Spectrum probable structure	*m/z* (relative intensity)			
M^+	407(0.3)	461(0.4)	515(0.6)	485(0.3)
$M-CH_3$	392(2.3)	446(7.0)	500(10)	470(8.4)
M−HCl	371(3.7)	425(4.8)	—	—
$M-CH_2Cl$	358(3.8)	412(3.4)	—	—
M−HCl	335(17)	—	—	—
$M-CH_2O(CH_3CO)$	—	—	—	412(9.4)
$M-Si(CH_3)_3OH$	—	371(1.8)	425(1.5)	395(2.8)
$M-CH_2OSi(CH_3)_3$	—	358(80)	412(100)	382(65)
$M-Si(CH_3)_3OH$—HCl	—	335(6.4)	—	—
$M-CH_2OSi(CH_3)_3$—CH_2Cl	—	309(3.7)	—	—
B	267(7.5)	267(34)	267(8.8)	267(14)
A	154(100)	208(100)	262(62)	232(100)
A−HCl	118(15)	—	—	—
$C_4H_7O_2^+$	—	—	—	87(70)
$Si(CH_3)_3^+$	73(16)	73(77)	73(19)	73(66)

to completion yielded, by titration, the theoretical amount of chloride ion. The hydrolysis products, II and III, were identified as their trimethylsilyl derivatives (IIa and IIIa) by GC–MS analysis of a typical hydrolysis mixture.

Stability *Versus* pH by Chloride Titration—The hydrolysis of I in 10% dimethylacetamide and sodium citrate buffer (4.2×10^{-2} *M*) as a function of pH between pH 3.0 and pH 6.0 is shown in Fig. 3. The percent of chlorine remaining covalently bound in I and II is calculated based on the percent of the theoretical amount of chloride that has been liberated at a given time.

The liberation of chloride from I is a function of pH of the media as seen in Fig. 3. The rate of hydrolysis increases as the pH increases. The slower rate at lower pH may be due to the protonation of the nitrogen of the alkylating function which inhibits the formation of the postulated aziridinium ion intermediate (Scheme I). Over the pH range studied (3.0–6.0) the rate of hydrolysis is too rapid for practical application.

Stability *Versus* pH by GLC—Table I compares the percentage of total chloride liberated from I in 0.042 *M* ammonium acetate buffer at pH 4.0 as determined by immediate direct titration and as calculated on the basis of the amounts of I, II, and III present determined by GLC analysis. These data indicate that the required manipulations prior to GLC analysis (freezing, freeze-drying, and derivatization) did not produce additional hydrolysis of I. Similar data demonstrate the comparability of the two methods in the pH stat controlled experiments. However, in this instance, it was necessary to apply corrections to the titration data to compensate for chloride bleed from the pH electrode.

The concentrations of I, II, and III *versus* time at pH 4.0 (pH stat control) as determined by GLC are seen in Fig. 4. Trimethylsilylation was essential for forming volatile derivatives with good GC properties from the degradation products of I. Combined GC–MS analysis showed that the hydantoin nitrogen as well as any free hydroxyl groups were derivatized by *N,O-bis*(trimethylsilyl)trifluoroacetamide. Table II lists the isothermal retention indexes of these derivatives and summarizes their mass spectral fragmentation patterns. The predominant mode of fragmentation was cleavage at bonds α to the mustard nitrogen with charge retention on this nitrogen. When the ethylene moiety between the hydantoin ring and mustard nitrogen was cleaved, Fragment A resulted as a major ion. Cleavage of the other C—C bonds α to the mustard nitrogen resulted in loss of substituted methyl radicals to form abundant ions such as *m/z* 358 in IIa, *m/z* 412 in IIIa, and *m/z* 382 in IVa. Cleavage of the bond adjacent to the mustard nitrogen and charge retention on the hydantoin moiety produced Fragment B at *m/z* 267, which was found in the MS of every derivative. The presence or absence and number of chlorine atoms was also evident from the distinctive isotopic pattern of this element. This information, plus an indication of the molecular weight from the molecular ion and fragmentation due to neutral losses (*e.g.*, $M-CH_3$, M−HCl), was more than sufficient to determine the identity of these derivatives.

The disappearance of I was pseudo first-order as indicated by the linearity ($r > 0.998$) of plots of the log of concentration of I *versus* time at pH 4.0, 5.0, and 6.0 (pH stat control). Pseudo first-order rate constants (k_1) at pH 4.0, 5.0, and 6.0 were 0.0336, 0.0472, and 0.0492/min, respectively. This pH–hydrolysis rate relationship demonstrates the same trend seen with the chloride titration data (Fig. 3). If the hydrolysis of I is

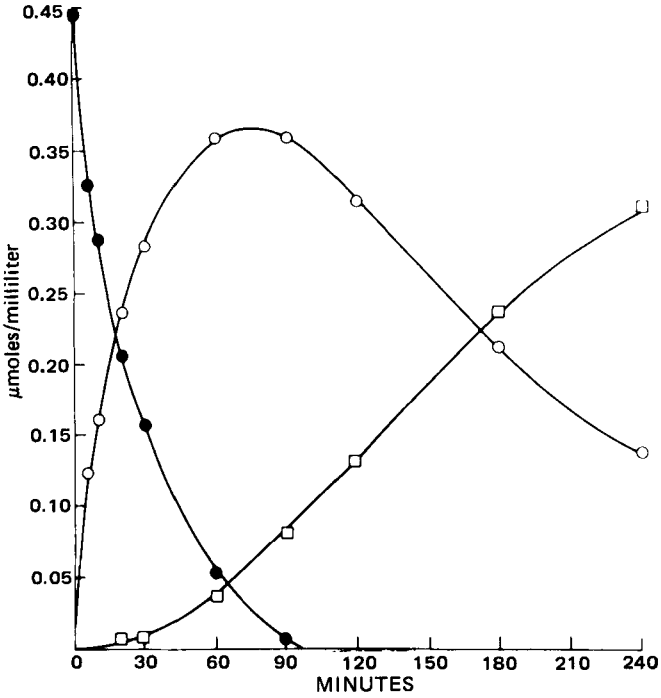


Figure 4—*Concentration* versus *time curves at pH 4.0 (apparent) and 25 ± 0.1°. Key: (●) I; (○) II; (□) III.*

represented by a mechanism comprised of consecutive pseudo first-order reactions (*i.e.*, $k_1 \gg k_{-1}$, $k_1 \ll k_2$, and $k_3 \gg k_{-3}$, $k_3 \ll k_4$),

$$I \xrightarrow{K_1} II \xrightarrow{k_3} III$$

where k_1 and k_3 are pseudo first-order rate constants, and it is possible to obtain estimates of k_3 by a method using dimensionless parameters and variables (5, 6). Application of this method gave $k_3 = k_1/14.0$ at pH 4.0 and $k_3 = k_1/1.9$ at pH 6.0.

Interpretation of Chloride Titration Data at Various pHs—The decomposition of I as a function of solution pH (Fig. 3) as determined by the titration of chloride ion shows a significant deviation from linearity in plots of percent chloride remaining $[\%(Cl_\infty^- - Cl_t^-)/(Cl_\infty^- - Cl_0^-)]$ *versus* time at ≤pH 4.0. To investigate any relationship these deviations might have with the protonation of I, II, or III, it was necessary to have information regarding their pKa values. The instability and limited solubility of I rendered pKa measurements impractical. However, it was possible to estimate by potentiometric titration with 0.05 *N* HCl a pKa of 6.4 for III. A spectrophotometric estimation of the pKa value for the dialcohol hydrolysis product of aniline mustard (NSC-18429) by a standard method (7) is 4.3. A published pKa for aniline mustard is 2.2 (8) indicating a 2.1-U difference between the pKa values of aniline mustard and its final hydrolysis product. If the aniline mustard–dialcohol model is roughly analogous the pKa of I may be ~4.3. The rate of hydrolysis of I or II decreases as pH decreases, presumably due to the protonation of the alkylating function nitrogen which inhibits aziridinium ion formation (an intermediate in the hydrolytic cleavage). A decrease in the rate of hydrolysis of I as pH decreases is reflected in the GLC data at pH 6.0, 5.0, and 4.0. If the pKa of II is taken to be intermediate between I and III the value would be 5.3. At pH 4.0 Compound II would be >95% protonated, which should significantly affect the hydrolysis rate (k_3) when compared to the rate at pH 6.0. This is supported by the data which indicate a sevenfold decrease in k_3 between pH 6.0 and 4.0. The decrease in the hydrolysis of I (k_1) between pH 6.0 and 4.0 is less dramatic, because with a pKa of 4.3 significant amounts of I remain unprotonated even at pH 4.0. The shape of the decomposition curves based on the release of chloride ion (Fig. 3) at ≤pH 4.0 are consistent with the lower rate (k_3) at which II releases chloride at low pH. The effect of various values of k_3/k_1 on the linearity of theoretical chloride ion plots have been described (9). It has also been shown (10) that an inverse relationship exists between hydrolysis rate and degree of protonation of a nitrogen mustard.

Chloride titration, although lacking specificity, is a useful tool in the investigation of the hydrolysis of nitrogen mustards. This is particularly true when used in a screening context (*e.g.*, to evaluate the effect of various solvent mixtures and other excipients on the hydrolysis rate).

Table III—Comparison of Stability of I to Melphalan and Mechlorethamine at 25°

Drug[a]	pH (apparent)	Time, Liberation of 50% Theoretical Cl^-, hr[b]
I	5.0	0.3
Melphalan	5.0	9.0
Mechlorethamine hydrochloride	5.0	8.4

[a] All drugs were 1×10^{-3} *M* in 10% dimethylacetamide–citrate buffer (4.2×10^{-2} *M*). [b] Determined graphically from a plot of log $(Cl_\infty^- - Cl_t^-)/(Cl_\infty^- - Cl_0^-)$ *versus* time.

However, particular care must be taken in the interpretation of chloride ion titration data derived from the hydrolysis of nitrogen mustards at pH near or between the pKa values for the intact mustard and its hydrolysis products. Since the titration of chloride is not a specific method (*i.e.*, the disappearance of I is not measured independently), the rate of chloride ion release measures the combined effect of both chloride generating reactions. Therefore, it is important to be aware of the effect of the relative magnitudes of k_1 and k_3 when interpreting such data.

Comparisons to Other Mustards—A comparison of the rates of hydrolysis as indicated by the liberation of chloride at pH 5.0 of I and two clinically useful mustards, mechlorethamine hydrochloride (NSC-762) and melphalan (NSC-8806), is presented in Table III. Compound I is much less stable than either of these mustards. The aromatic nitrogen mustards (melphalan and the aromatic hydantoin mustards described previously) (11) are inherently more stable due to the decreased basicity of the alkylating function nitrogen when attached to an aromatic ring. The apparent superior stability of mechlorethamine hydrochloride relative to I (Table III) is probably because of the fact that its more stable hydrochloride salt is formed (the formation of the aziridinium ion intermediate is blocked) and can be administered at a pharmaceutically acceptable pH (3.0–5.0 for the reconstituted USP product) (12). In contrast, the hydrochloride salt of I could be isolated only at pH <1 and upon storage was unstable[21]. Salt formation was effective for increasing solubility only at very low, pharmaceutically unacceptable pH values. Subsequent upward adjustment of pH caused precipitation of I.

Attempts to Stabilize I—The stability of I in the presence of a chemically diverse group of additives is seen in Table IV. Significant increases in the stability of some aromatic nitrogen mustards in the presence of surface active agents has been shown (13). Several surfactants slowed the hydrolysis of I (Table IV) with the best, stearylcetyl alcohol ethoxylate[5], increasing the half-life of I six- to sevenfold at pH 5.0. The increases in the stability of I appear to parallel the lipophilicity of the surface active agents. At lower pH (3.0 and 4.0) in the presence of 5% polyoxyethylated castor oil[3] the relative increases in stability of I are less. This diminished effect is probably due to a decreased solubility of I in the micelle as more drug is protonated. Solutions of up to 30% ethanol or 50% propylene glycol have no significant effect on the hydrolysis of I. Some mustards were found to be more stable in the presence of albumin (13, 14). However, the hydrolysis of I was very rapid in the presence of 5% albumin.

The disappearance of I as monitored by GLC at pH 4.0 in the presence of 0.15 *M* NaCl demonstrates the inhibitory effect of chloride ion on the hydrolysis of I. The pseudo first-order rate constant for the disappearance of I under these conditions is 0.00925/min. This reduced hydrolysis rate is not unexpected, since it has been shown previously with other nitrogen mustards using specific assay procedures (15, 16). None of these approaches has dramatically increased the stability of I.

10% Fat Emulsion System—The 10% fat emulsion system combines two approaches which have increased the stability of I (*i.e.*, isolation of I from the aqueous component of the vehicle as with the surfactants and inhibition by the presence of chloride ion).

The stability of I in a 10% fat emulsion was determined by GLC. Attempts to extract I directly from the 10% fat emulsion with ethyl acetate for GLC analysis were erratic with the recovery varying from 80 to 37% in the 1.0–0.2 mg/ml concentration range. Other extraction solvents gave similar results. However, the extraction of I with ethyl acetate from the residue remaining after freeze drying the 10% fat emulsion was more consistent. An extraction efficiency of 89.4 ± 3.3% was observed over the 1–0.15 mg/ml concentration range. Compounds II and III were not extracted under these conditions. The relative standard deviation for the overall extraction GLC procedure was 5.0% on the basis of 10 extracted samples (0.7 mg/ml of I). Determination of the concentration of I by GLC in the emulsion system with subsequent filtration through a 0.45-μm filter and reassay indicated that the drug does not precipitate at 1 mg/ml. However, at 2 mg/ml considerable precipitation had occurred.

[21] J. S. Driscoll, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, personal communication, 1979.

Table IV—Stability of I in Various Media at 25 ± 0.1°

pH (apparent)	Media[a]	Time, Liberation of 50% Theoretical Cl^-, hr[b]
	Buffers	
5.0	4.2×10^{-2} *M* citrate	0.3
4.0	4.2×10^{-2} *M* citrate	0.9
3.5	4.2×10^{-2} *M* citrate	0.9
3.0	4.2×10^{-2} *M* citrate	2.5
	Surfactants	
5.0	5% polysorbate 20[c]	0.7
5.0	5% polysorbate 80[d]	0.9
5.0	5% polyoxyethylated castor oil[e]	1.1
5.0	5% stearylcetyl alcohol ethoxylate[f]	1.6
5.0	5% polyoxyethylated vegetable oil[g]	0.9
4.0	5% polyoxyethylated vegetable oil[g]	1.2
3.0	5% polyoxyethylated vegetable oil[g]	1.5
	Solvents	
5.0	30% ethanol	0.6
5.0	50% propylene glycol	1.0
4.0	30% propylene glycol	0.6
3.5	50% propylene glycol	1.1
3.5	50% polyethylene glycol 400	1.2
	Miscellaneous	
7.0	5% albumin	0.3

[a] In addition to the component listed, each solution contained 10% dimethylacetamide and sodium citrate buffer 4.2×10^{-2} *M*. [b] Graphically estimated from a plot of log $(Cl_\infty^- - Cl_t^-)/(Cl_\infty^- - Cl_0^-)$ *versus* time. [c] Tween 20. [d] Tween 80. [e] Cremophor El. [f] Siponic E-7. [g] Emulphor EL-719.

The disappearance of I in 10% fat emulsion was monitored by GLC. Plots of the concentration of I *versus* time were linear ($r > 0.99$). The release of I from the fat component of the emulsion may be the rate-limiting step.

A fat emulsion identical to the one used in this report has been used as a vehicle for another unstable antitumor agent (17). Also, large volumes of the fat emulsion alone have been used clinically as a nutritional source (17, 18). A projected human dose for I derived by the application of equivalent surface area dosage conversion factors described previously (19) to the mouse antitumor data would indicate a dose of 6–48 mg for a 60-kg human. This would require the administration of 6–48 ml of the 10% fat emulsion formulation (1 mg/ml of I). A formulation of another antitumor agent in 10% fat emulsion has been given at 1 ml/min with no adverse effects (17). At the time of use, a vial containing I produced by low temperature vacuum drying (20) would be dissolved in dimethylacetamide with the resulting solution being added to the fat emulsion as described in the Experimental section. Administration of appropriate doses of the formulation of I could be accomplished by an infusion at 1 ml/min or slightly faster. This would allow the entire dose to be administered before unacceptable decomposition (≥10%) occurred.

Biological Activity—The antitumor activity of I in the emulsion formulation was compared with a suspension of I *versus* intraperitoneally implanted P-388 leukemia cells in three experiments. The antitumor response of I in both emulsion and suspension preparations was comparable.

Summary—The hydrolysis of I is quite rapid. Several attempts (Table IV) to increase the stability of I have provided less than dramatic results. Although the 10% fat emulsion system has significantly stabilized I, the decomposition rate remains rather rapid resulting in a formulation that is only marginally acceptable. It is unfortunate that I exhibits poor solubility in addition to the instability. Other nitrogen mustards are inherently more stable, such as the aromatic mustards (*e.g.*, melphalan) or they are more soluble allowing for more rapid administration in a small volume (*e.g.*, mechlorethamine).

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ACKNOWLEDGMENTS

The authors thank Mr. Douglas J. Wiederrich for technical assistance and Dr. Randall K. Johnson, Arthur D. Little, Inc., Cambridge, Mass. for performing the tumor studies.

The Use of *N,N*-Diethyl-*m*-Toluamide to Enhance Dermal and Transdermal Delivery of Drugs

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Abstract □ A dermal penetration enhancer has been found which improves the dermal delivery of a wide variety of drugs and at the same time has a history of low toxicity for human dermal application. *N,N*-Diethyl-*m*-toluamide (I) has been shown to improve the delivery of many drugs through hairless mouse skin in an *in vitro* diffusion cell model. A topically applied steroid, hydrocortisone, has been used to demonstrate the *in vivo* effectiveness of I on human skin. The degree of pallor produced on human skin by the corticosteroids was used as a measure of the relative delivery of hydrocortisone from formulations with and without I.

Keyphrases □ *N,N*-Diethyl-*m*-toluamide—enhancement of dermal and transdermal drug delivery, hydrocortisone □ Hydrocortisone—enhancement of *N,N*-diethyl-*m*-toluamide on drug delivery, dermal and transdermal delivery □ Delivery, drug—dermal and transdermal, *N,N*-diethyl-*m*-toluamide enhancement, hydrocortisone

Improved dermal delivery of drugs has been the focus of pharmaceutical research worldwide for many years. The goal in most cases has been to find a substance of low toxicity which is nonirritating and will deliver a wide variety of compounds effectively.

Efforts to improve dermal delivery of drugs have included traditional formulation approaches and studies on the effects of surfactants, fatty acids, and glycols (1, 2). Although these approaches attained some degree of success, in no case was the enhancement of drug delivery spectacular. In contrast, dimethyl sulfoxide has been shown to greatly enhance dermal and transdermal delivery of a wide variety of drugs (3–11). Unfortunately, the use of this substance has been limited in humans to the treatment of interstitial cystitis by intravesical instillation. Dimethylacetamide has been found to enhance the delivery of a number of drugs for the treatment of skin diseases (12–14). However, the lack of long-term safety information has limited the use of this compound.

In the present study, the effects of *N,N*-diethyl-*m*-toluamide (I) on skin permeability was examined for a wide variety of drugs. Formulations of I have been used extensively as insect repellents. The compound was first reported to be an effective insect repellent in 1954 (15) and has been applied *ad libitum* to the skin in concentrations ranging from 10 to 100%. Despite prolonged and widespread use in humans, major side effects due to the penetration enhancer itself have not been encountered.

EXPERIMENTAL

Reagents and Drugs—Hydrocortisone[1], hydrocortisone acetate[1], hydrocortisone 17-butyrate[2], and hydrocortisone 17-valerate[3] were among the compounds used. Also used were dibucaine[1], benzocaine[4], indomethacin[1], ibuprofen[5], erythromycin[6], tetracycline hydrochloride[7], griseofulvin[8], mycophenolic acid[9], and methyl salicylate[10]. Triethanolamine salicylate was prepared by adding equal molar amounts of

[1] Sigma Chemical Co.
[2] Analysis calculated: C, 70.24; H, 8.16. Analysis found: C, 70.20; H, 8.12. Assay by HPLC to contain <2% hydrocortisone-21-butyrate.
[3] Analysis calculated: C, 69.96; H, 8.52. Analysis found: C, 69.69; H, 8.50.
[4] ICN Pharmaceuticals, Inc.
[5] Industrie Chemicle Farmaceutiche Italiane SpA.
[6] Assay 959 μg/mg, Sigma Chemical Co.
[7] Solid supplied with Topicycline.
[8] Ayerst Laboratories.
[9] Eli Lilly and Co.
[10] Matheson Coleman and Bell.

0022-3549/82/1100-1211$01.00/0