Reduction of Blue Tetrazolium by Corticosteroids

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Abstract ☐ Pseudo-first-order rate constants were observed for the reaction of corticosteroid or corticosteroid esters with blue tetrazolium. The data indicate that the reactivity of corticosteroids is, in part, a function of their geometry in that corticosteroid reactivity toward blue tetrazolium increases with increasing planarity of the steroid molecule and that corticosteroid esters must be hydrolyzed as a necessary prerequisite to reaction with blue tetrazolium. Evidence is presented indicating that free radicals are not involved in the blue tetrazolium reaction with corticosteroids. Certain pharmaceutically important compounds such as pyrocatechol derivatives and hydroquinone appear to reduce blue tetrazolium by the anion free radical mechanism proposed previously. A spectrophotometric method for determining the number of reduction units transferred to blue tetrazolium per molecule is described.

Keyphrases ☐ Blue tetrazolium—reduction by corticosteroids, pseudo-first-order rate constants, mechanism discussed ☐ Corticosteroids—reduction of blue tetrazolium, pseudo-first-order rate constants, mechanism discussed ☐ Steroids—reaction of corticosteroid and corticosteroid esters with blue tetrazolium, mechanism

The blue tetrazolium reaction (1) is widely used for the analysis of corticosteroids and is, with slight modification, the official procedure [USP XVIII (2) and NF XIII(3)]. In very basic alcoholic solution, the C_{17} side chain of the corticosteroid is oxidized and blue tetrazolium (I) is reduced to a colored formazan whose concentration is measured spectrophotometrically. Extensive investigations (4–16) of the reaction conditions established that the analytical procedure is subject to many variables, which are minimized by analyzing blank, standard, and sample solutions concurrently.

Kinetic and mechanistic studies, although not extensively investigated, revealed certain important aspects of the blue tetrazolium reaction. For example, the rate of the reaction of cortisone and hydrocortisone with I was found to be first order in the corticosteroid (17). Of these two corticosteroids, cortisone was observed to be the more reactive toward I.

In that study, it was also established that ester hydrolysis of cortisone acetate was a prerequisite to reaction with I. Significant variations in the reaction rates of corticosteroids of closely related structures with I have been noted (6, 13–15, 18).

Meyer and Lindberg (18) established that the α-ketol moiety in corticosteroids and other α-ketol-containing compounds is responsible for the reduction of I and that the electron-donating ability of substituent groups may be correlated with the rate of formazan development.

Sinsheimer and Salim (19) showed that some nonketol compounds such as polyhydric phenols, quinones, and certain active hydrogen compounds are sufficiently reactive to permit quantitative analysis *via* formazan formation. They proposed a free radical mechanism based on similar structural reactivity in the blue tetrazolium reaction to the reaction of the same compounds toward oxygen.

This paper shows that the reduction of blue tetrazolium by corticosteroids and several other compounds occurs as a unit function. The difference in reaction rates of several corticosteroids is related to their differences in structure. The results are consistent for the 25 steroids studied.

EXPERIMENTAL

Apparatus—The following were used: a UV–visible ratio recording spectrophotometer[1] with 1-cm quartz cells, an electrobalance[2], a computer[3], an electron-spin resonance instrument[4], and a TLC apparatus[5].

Reagents—Alcohol USP, absolute methanol, acetic acid, and 50% hydrogen peroxide were all analyzed reagent grade. The 1% tetramethylammonium hydroxide (II) was prepared by diluting 5.00 ml of 10% aqueous[6] II to 50.0 ml with alcohol USP (95% ethanol). Solutions of iodine, 0.010 mg/ml in alcohol USP, and blue tetrazolium[7], 5.0 mg/ml in absolute methanol, were prepared. Corticosteroid standard solutions contained 0.010 mg/ml in alcohol USP unless otherwise indicated.

General Procedure—The procedure followed, unless otherwise specified, is the official procedure given in USP XVIII (2), in which a 20.00-ml volume of standard or sample corticosteroid in alcohol USP is treated with 2.00 ml of I reagent (5 mg/ml), followed by 2.00 ml of 1% II. The absorbance is measured against a reagent blank 90 min after the addition of II.

Corticosteroid Decomposition by II—Duplicate 20.0-ml aliquots of betamethasone, betamethasone 17-benzoate, betamethasone 21-benzoate, and dichlorisone acetate were pipetted into glass-stoppered flasks, and 2.00 ml of 1% II was added to one set. Both sets were then stoppered and placed in the dark for 24 hr. Two milliliters of I (5 mg/ml) were added to the set to which II had been added, and the solution was scanned for 90 min after the addition of I. The general procedure was followed in the other set.

Effect of Water on Blue Tetrazolium Reagent-.Five-milliliter aliquots of I were diluted 1:1 with water or with alcohol USP and allowed to stand for 16 hr. A blank and triplicate aliquots of hydrocortisone were run in accordance with the general procedure except that the diluted I was used.

Oxygen Effect—Six 20.0-ml aliquots of alcohol USP were pipetted into 25-ml glass-stoppered flasks. Oxygen was bubbled through two aliquots and simultaneously through glass-stoppered cylinders containing I and II for 20 min. This procedure was repeated for two other aliquots using nitrogen instead of oxygen. The remaining two air-saturated aliquots were used as controls.

All six aliquots were run by the general procedure, except that the saturated samples of I and II were used with the respective saturated aliquots and all samples were scanned against alcohol USP instead of a reagent blank.

Electron-Spin Resonance Study—Cortisone (0.100 mg/ml in alcohol USP) was treated by the general procedure. Immediately after the addition of 1% II, the sample was scanned in an electron-spin resonance spectrometer. After the scan, the solution was

[1] Cary model 15.
[2] Cahn model G-2.
[3] Digital Equipment Corp. PDP/8e and IBM 360 model 25.
[4] Varian model 4502-13.
[5] Eastman 6060 silica gel with fluorescent indicator.
[6] Eastman Organic Chemicals.
[7] Dajac Laboratories.

Table I—Decomposition of Selected Corticosteroids by Tetramethylammonium Hydroxide

Corticosteroid	Source[a]	Amount, mg	Absorbance: Decomposed Steroid[b]	Absorbance: Control	Decomposition[c], %
Betamethasone	1	0.202	0.195	0.479	59
Betamethasone 17-benzoate	2	0.241	0.193	0.422	54
Betamethasone 21-benzoate	2	0.243	0.187	0.414	55
Dichlorisone acetate	3	0.203	0.078	0.453	83

[a] 1 = NF Reference Standard; 2 = Warner Lambert Co., Morris Plains, N.J.; 3 = Division of Pharmaceutical Sciences, Food and Drug Administration, Washington, D.C. [b] Steroid plus 1% II was mixed for 24 hr before quantitation. [c] Calculated as:

$$100 - \frac{\text{absorbance of decomposed steroid}}{\text{absorbance of control}} \times 100$$

swept with nitrogen gas for 20 min and rescanned for 40 min from the time of addition of 1% II. Cortisone (1.00 mg/ml in alcohol USP) was treated similarly, except that the second scan occurred 90 min after the addition of 1% II. Scans were also made of alcohol USP, alcohol USP plus I, and alcohol USP plus I and 1% II.

Rate Studies—A 20.0-ml aliquot of a standard and a 20.0-ml blank of alcohol were treated by the general procedure, with zero time taken as the time of the addition of 1% II to the standard solution. Both solutions were transferred to cells as rapidly as possible and placed in the spectrophotometer, and readings were made each minute at 525 nm until the reaction reached essential completion.

The procedure was repeated for a total of 25 different corticosteroids. Rate constants were calculated by a computer program. This procedure was repeated for several noncorticosteroid steroids and other selected pharmaceutical compounds.

Hydrolysis of Betamethasone 17-Benzoate—Duplicate 20.0-ml aliquots of the betamethasone and betamethasone 17-benzoate standards were treated with 2.00 ml of 1% II and allowed to stand for 75 and 135 min, respectively; the reaction was terminated by the addition of 1.00 ml of acetic acid. The solutions were washed into a separator with 75 ml of water and extracted five times with 20.0-ml portions of chloroform.

Each extract was washed through a second separator containing 15 ml of water and filtered through chloroform-washed cotton, and the combined extract was taken to dryness carefully under air on the steam bath. The residues were dissolved in 0.2 ml of methanol, and 1 µl was spotted with standards at the same concentrations on a TLC plate and developed with chloroform–methanol (36:1).

Effect of Free Radical Reaction Inhibitor—One set of 10.0-ml aliquots of hydrocortisone (0.020 mg/ml in alcohol USP), fluoxymesterone (0.020 mg/ml in alcohol USP), hydroquinone (0.010 mg/ml in alcohol USP), and isoproterenol sulfate (0.006 mg/ml in alcohol USP) was mixed with 10.0 ml of iodine reagent, and another set was mixed with 10.0 ml of alcohol USP. Both sets were run *versus* a blank by the procedure discussed under *Rate Studies.*

RESULTS AND DISCUSSION

Experiments designed to determine the extent of reactions between corticosteroids and/or formazan with the ingredients of the reaction medium were carried out with typical compounds before rate studies were attempted.

Table I shows the results of the study of the interaction of a typical corticosteroid and corticosteroid esters with II. Since both the corticosteroid and its esters were partially decomposed after 24 hr of contact with II, the reaction of II evidently occurs also at the C_{17} side chain and competes with I during the usual 90-min reaction time. For the longer periods needed in some of the rate studies, increased base degradation and less formazan formation are expect-

Table II—Rate Constants and Absorbance per Micromole for Selected Corticosteroids

	Source[a]	T_i[b], min	A'[c]	k[d]	Relative Rate[e]	Time to A_{max}, min	Maximum Absorbance	Absorbance per Micromole
Betamethasone	1[f]	71	0.300	0.024	3.2	370	0.460	0.894
Flurandrenolide	4	40	0.454	0.056	7.4	90	0.597	1.161
19-Norhydrocortisone	6	40	0.515	0.072	9.5	90	0.597	1.012
Hydrocortisone	5	34	0.545	0.090	12	60	0.596	1.076
Prednisolone	5	34	0.548	0.092	12	130	0.610	1.092
Triamcinolone	5	38	0.999	0.093	12	90	1.112	2.193
11-Desoxycortisone	7	36	0.548	0.094	12	90	0.572	0.990
Methylprednisolone	1[f]	28	0.502	0.096	13	70	0.549	1.007
5-Pregnen-3β,21-diol-20-one	7	33	0.479	0.096	13	90	0.516	0.857
Corticosterone	7	32	0.531	0.098	13	90	0.579	1.010
Dexamethasone	1[f]	34	0.519	0.098	13	50	0.544	1.069
11-Desoxycorticosterone	7	31	0.575	0.109	14	90	0.616	1.011
Fluprednisolone	1[f]	25	0.497	0.121	16	61	0.547	1.034
Cortisone	8	16	0.557	0.243	32	90	0.583	1.045
Dihydrocortisone	7	18	0.560	0.257	34	60	0.566	1.017
Prednisone	5	13	0.556	0.305	40	31	0.568	1.012
Betamethasone 17-benzoate	2[f]	90	0.130	0.0076	1	394	0.441	0.913
Betamethasone 21-benzoate	2[f]	90	0.133	0.017	2.2	470	0.438	0.878
Hydrocortisone acetate	5	36	0.479	0.081	11	60	0.520	1.046
Prednisolone acetate	5	33	0.473	0.086	11	90	0.517	1.030
11-Desoxycorticosterone acetate	5	33	0.499	0.090	12	60	0.563	1.048
Dichlorisone acetate	3[f]	29	0.451	0.102	13	90	0.477	1.070
Dihydrocortisone acetate	7	20	0.490	0.200	26	40	0.503	1.009
Cortisone acetate	5	18	0.501	0.230	30	90	0.528	1.056
Prednisone acetate	7	15	0.490	0.307	40	90	0.497	0.998

[a] 4 = Eli Lilly and Co., Indianapolis, Ind.; 5 = USP Reference Standard; 6 = Syntex Research, Palo Alto, Calif.; 7 = Schwarz/Mann, Orangeburg, N.Y.; 8 = K and K Laboratories, Inc., Plainview, N.Y. [b] T_i is time in minutes of observation for calculation of rate constant. [c] A' is optimum absorbance for calculation of rate constant. [d] k is rate constant. [e] Compared to betamethasone 17-benzoate, which has the slowest rate. [f] See Table I for source.

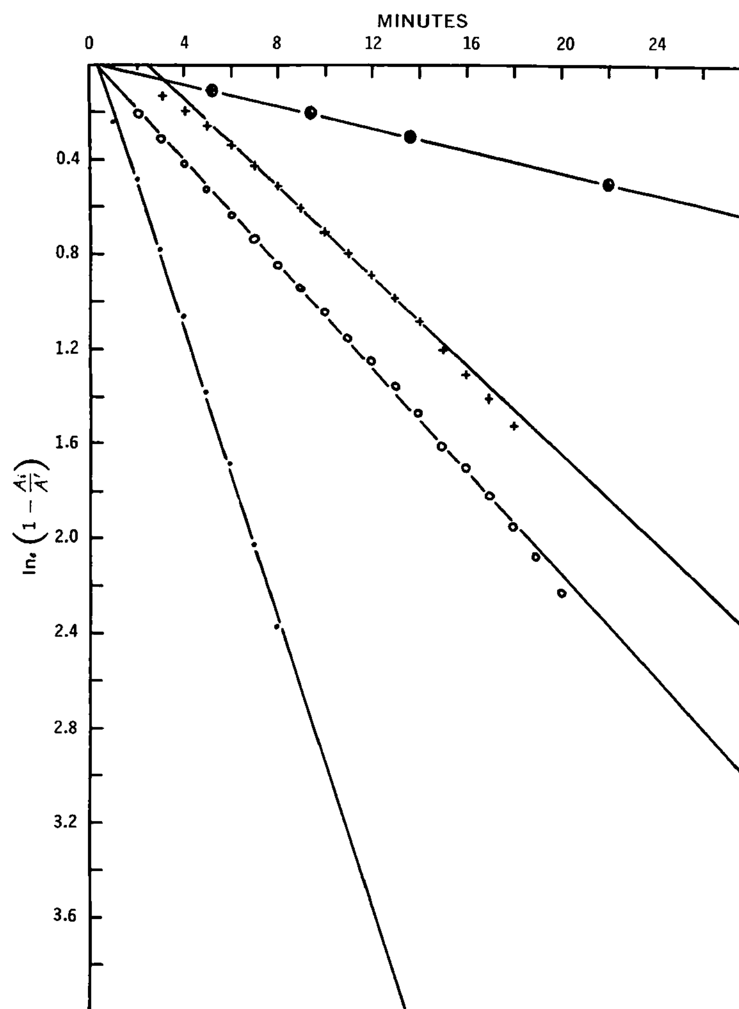

Figure 1—*Reaction rates of typical corticosteroids. Key: ⊕, betamethasone; +, 11-desoxycorticosterone acetate; ○, 11-desoxycorticosterone; and ●, prednisone.*

ed. As an example, the time necessary to attain a maximum absorbance of 0.460 for betamethasone is 370 min, whereas an equimolar amount of flurandrenolide reaches a maximum absorbance of 0.597 in 90 min.

Triplicate aliquots of hydrocortisone run by the general procedure, using a 1:1 dilution of the solution of I prepared for the study with water, gave absorbances of 0.561, 0.561, and 0.562, while the control group with a 1:1 dilution of the same solution of I with alcohol USP gave absorbances of 0.577, 0.577, and 0.576. The results indicate that water does not cause decomposition of I; the 3% decrease in sensitivity probably is due to the apparent pH effect of water (16).

The results of the study of the effect of oxygen on the blue tetrazolium blank showed that the average net absorbance of duplicates run *versus* alcohol USP as the blank was 0.070 for the air-saturated control, 0.078 for the oxygen-saturated solutions, and 0.084 for the nitrogen-saturated solutions. Under the conditions studied, oxygen apparently does not contribute to the blank in the blue tetrazolium reaction.

In addition, the blue tetrazolium–corticosteroid reaction was monitored by electron-spin resonance. The electron-spin spectrum showed the presence of an unpaired electron, which was a function of the concentration of the cortisone used and which did not change during the 90 min required for the reaction of cortisone with I to go to completion. This indicates that the unpaired electron was not involved in the production of formazan.

The results of the rate study of the blue tetrazolium reaction with corticosteroids are shown in Table II. Pseudo-first-order rate constants were observed for all 25 corticosteroids. The rate constants were obtained by performing a least-squares regression on the following equation:

$$\ln_e\left(1 - \frac{A_i}{A'}\right) = kT_i + E \qquad \text{(Eq. 1)}$$

where A_i is the absorbance at any time (T_i), A' is the optimum absorbance, k is the rate constant, and E is the intercept. The intercept for a first-order equation should be zero, but the intercept of the regression line is shifted due to the time lag caused by the addition of the last reagent. The index of determination (R^2) for the regressions was greater than 0.99 in most cases.

The rate constants listed in Table II range from 7.6×10^{-3} for betamethasone 17-benzoate to 3.07×10^{-1} for prednisone acetate, which indicates that prednisone acetate reacts 40 times faster than betamethasone 17-benzoate.

The reaction of I with 0.55 μmole of hydrocortisone in the presence of 0.41 μmole of iodine showed an absorbance of 0.412 after 22

Table III—Reduction Units Furnished by Noncorticosteroid Pharmaceuticals

Compound	Source[a]	Sample, μmoles	Reaction Time	Maximum Absorbance	Absorbance Per Micromole	Number of Reduction Units[b]
Hydroquinone	9	0.1101	5 hr	1.107	1.179	1.14
Epinephrine bitartrate	8[c]	0.2934	10 min	0.931	3.173	3.08
Isoproterenol sulfate	3[c]	0.0692	30 min	0.281	4.061	3.94
Isoproterenol hydrochloride	5[c]	0.2077	40 min	0.784	3.774	3.66

[a] 9 = Fisher Scientific Co., Fairlawn, N.J. [b] Calculated as 1.031 absorbance units equals 1 reduction unit. [c] See previous tables for source.

Table IV—Molar Absorptivity of Selected Compounds

Compound	Source[a]	Absorbance	Concentration, g/liter	Molecular Weight	Molar Absorptivity[b]	Number of Reduction Units
Hydrocortisone	5	0.596	0.01004	362.47	21,500	1
Hydroquinone	9	1.070	0.004995	110.11	23,600	1
Triamcinolone	5	1.112	0.0100	394.45	43,900	2
Epinephrine bitartrate	8	0.931	0.004890	333.29	63,500	3
Isoproterenol hydrochloride	5	0.784	0.002573	247.70	75,600	4
Isoproterenol sulfate	3	0.281	0.001008	291.31	81,200	4

[a] See previous tables for source. [b] Maximum error is 1.75% as determined by National Bureau of Standard Calibration.

min, while the absorbance of a duplicate without iodine was 0.408 for the same time. Evidently, the reaction rate of hydrocortisone with I is not inhibited by iodine, even though iodine is a radical scavenger which inhibits radical reactions. This failure to inhibit suggests that the oxidation of corticosteroids by I does not proceed by a free radical mechanism.

The reactivity of the corticosteroids toward I appears to be, at least in part, a function of their molecular shape. Steroids with C_{16} substitution in either the α or β position react the slowest, steroids that possess a planar A ring and have sp^3 C_{11} substitution show intermediate reactivity, and steroids possessing a C_{11} carbonyl react the fastest with I. Thus the more planar the steroid molecule, the faster is the reaction with I.

This trend, coupled with the electron-spin resonance experiment, is evidence that a bimolecular reaction between I and the corticosteroid occurs in which a reduction unit from the ketol group is transferred from the steroid to I to form the formazan. In addition, the approach of the blue tetrazolium molecule occurs presumably at the β face of the steroid since greatest rate retardations are observed for steroids having β substitution near the ketol group (*e.g.*, C_{16}).

The rates of reaction of several corticosteroid esters with I were also studied (Table II). Graphical representation of typical kinetic data (Fig. 1) indicates that ester hydrolysis occurs prior to and during the early stages of formazan development, thus substantiating Guttman's (17) similar observations in the case of cortisone acetate. In addition, TLC analysis of a partially completed (25%) reaction mixture of betamethasone 17-benzoate and I revealed that the ester had been completely converted to betamethasone and its base decomposition products.

The order of reactivity of the esters listed in Table II parallels that of the corresponding corticosteroids toward I. Overall reaction rate constants are listed in Table II. The deviation between the rate constant for corticosteroids and their corresponding esters other than betamethasone reflects the generally small contribution of the hydrolysis rate to the overall reaction rate constant, k.

Calculation of the absorbance per micromole of most of the corticosteroids listed in Table II yields an average value of 1.031 ± 0.056, which corresponds to the transfer of one reduction unit. The three betamethasones were not included in the average since the long period required for their reactions with I resulted in low absorbance values due to extensive basic decomposition of the steroid.

The absorbance per micromole value (2.193) for triamcinolone suggests that two reduction units are transferred per molecule of triamcinolone. Transfer of the second reduction unit probably arises from the formation of a second α-ketol group during the base degradation of the initially formed glyoxal derivative (20, 21), which contains both C_{16} hydroxyl and C_{17} carbonyl groups.

Several pharmaceutically important compounds known to reduce I (22) were tested (Table III). These results indicate that hydroquinone, epinephrine, and isoproterenol sulfate reduce 1, 3, and 4 equivalents of I, respectively. The high reduction value for hydroquinone (1.14 units) may be due to oxidative cleavage of the quinone ring after the reduction is complete, as suggested by Sinsheimer and Salim (19).

The reaction with epinephrine bitartrate was complete in 10 min and thus was not subject to basic degradation by II under the conditions of the general procedure. Also, an aliquot of alcoholic epinephrine bitartrate produced no adrenochrome when treated with II for 24 hr, but addition of I to this solution after 24 hr produced a formazan with typical spectrum. This finding suggests that the absorbance value of 1.058/reduction unit is close to the true value. The reaction of I with hydroquinone and with isoproterenol sulfate was greatly inhibited by the presence of iodine, which indicates that the anion intermediate radical mechanism proposed (19) takes place in these systems.

The results of the study of the effect of 0.41 μmole of iodine on the reduction of I by 0.59 μmole of fluoxymesterone by the general procedure showed no inhibition of the rate of reaction during the first 30 min or after 20 hr when compared with an equimolar solution of fluoxymesterone containing no iodine. Androst-4-ene-3,17-dione-type steroids have been shown (18) to reduce I. In that study, androst-4-ene-3,17-dione was observed to be oxidized by I to a C_6 carbonyl derivative *via* the corresponding C_6 alcohol intermediate.

Table IV shows molar absorptivity values calculated by the method given in USP XVIII (2, p. 825). It appears that there is a linear relationship between the molar absorptivity and the number of reduction units involved in the reaction, with the molar absorptivity being approximately 20,000 for each unit.

The nature of the formazan produced in these reactions and the effect of solvent on the rate of reaction of I with various steroids and pharmaceutically important compounds are currently being investigated.

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Reactions of 2,2-Dimethylaziridine-Type Alkylating Agents in Biological Systems II: Comparative Pharmacokinetics in Dogs

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Abstract ☐ Pharmacokinetic studies in dogs were performed with a series of three 2,2-dimethylaziridine antineoplastic agents. These alkylating agents were relatively unstable in all aqueous media, with half-lives of 9–15 min in whole canine blood. This finding indicates that the aziridine compounds are capable of undergoing destruction in all biological tissues. A pharmacokinetic analysis of the plasma concentration *versus* time data was performed using a two-compartment open model, which includes drug elimination from both compartments. The three aziridine compounds showed similar pharmacokinetic behavior with respect to their loss from plasma, but they exhibited differences in their *in vivo* release of a potent alkylating intermediate (2,2-dimethylaziridine) and a second product, which was a strong inhibitor of pseudocholinesterase.

Keyphrases ☐ 2,2-Dimethylaziridines—reactions in biological systems, comparative pharmacokinetics in dogs ☐ Aziridines—2,2-dimethylaziridine-type alkylating agents, reactions in biological systems, comparative pharmacokinetics in dogs ☐ Antineoplastic agents—reactions of 2,2-dimethylaziridine-type alkylating agents in biological systems, comparative pharmacokinetics in dogs ☐ Pharmacokinetics—2,2-dimethylaziridine-type alkylating agents in biological systems, dogs

The 2,2-dimethylaziridine-type alkylating agents ethyl bis(2,2-dimethylaziridinyl)phosphinate (I), bis(2,2 - dimethylaziridinyl)phosphinylurethan (II), and tris(2,2-dimethyl-1-aziridinyl)phosphine oxide (III) have been shown to possess significant antitumor activity in a number of animal systems (1–3) and in humans (4–6). One characteristic chemical property is their unusually rapid hydrolysis with concomitant loss of their alkylating activity (7).

Since the effectiveness of an alkylating agent is probably a function of its reactivity toward various nucleophilic targets and of its local concentration and contact time with these targets, the *in vivo* activity of these particularly unstable alkylating agents is an interesting phenomenon and suggested that a study be made of the pharmacokinetic behavior of these drugs. The plasma concentration–time curves for such agents represent useful data for the scientific application of these drugs to the treatment of neoplastic diseases. Furthermore, a pharmacokinetic study of a series of agents with related structures could provide useful information for the design of new antineoplastic drugs.

This report describes the pharmacokinetic behavior of I–III in the dog following various routes of administration and the identification of 2,2-dimethylaziridine (IV) as part of the metabolic pathway of I and III in the plasma of dogs.

EXPERIMENTAL

Materials—The reagents used in the stability studies, pharmacokinetic studies, and analytical procedures were purchased and/or purified as described previously (7).

Analytical Procedures—The measurement of alkylating activity and the quantitative analysis of samples were performed with 4-(*p*-nitrobenzyl)pyridine, using the previously reported procedures (7).

Stability Studies—The rate of loss of I–III in various buffers, urine, plasma, and whole blood was studied. The methodology used was described previously (7).

Pharmacokinetic Studies—Male mongrel dogs, 20–28 kg, were fasted for approximately 18 hr before drug administration. The animals were lightly anesthetized with pentobarbital sodium, and one cephalic vein was catheterized for the collection of blood. Preliminary studies showed that the 2,2-dimethylaziridine compounds were stable in alkaline solution. Therefore, they were prepared for injection by dissolution in 5 ml of 0.05 M Na_2CO_3 (pH adjusted to 11.0) or 10^{-3} M KOH.

All three compounds were administered by intravenous injection, while II was also given by intramuscular injection into the gluteal muscle and by injection into the peritoneal cavity. Pretreatment with proadifen hydrochloride[1] [2-(diethylamino)-ethyl-2,2-diphenylvalerate hydrochloride] involved a dose of 20 mg/kg sc 1 hr before the administration of II.

Sample collection (sufficient blood to perform two to five determinations) was accomplished quickly (in about 20 sec) because of the rapid rate of change of plasma drug concentration. The time of sampling was assumed to be the midpoint of this brief collection interval. The catheter was maintained open by filling it with a heparin solution (100 units/ml in 0.9% NaCl). The contents of the catheter were discarded before each sample was drawn. The other mechanical features of sample handling were performed as described previously (7).

RESULTS

Stability Studies—The *in vitro* stability of I–III was measured in various buffers and biological media, using a broader varia-