Interaction of Mechlorethamine and Isophosphamide with Bovine Serum Albumin and Rat Liver Microsomes

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Abstract ☐ The interaction of the alkylating metabolites of iso-phosphamide and mechlorethamine with microsomal protein and bovine serum albumin was studied. The procedure of freezing and thawing of rat liver microsomal fraction greatly increases the inhibition by mechlorethamine of its ethylmorphine demethylase activity. Microsomal metabolites of isophosphamide bind covalently to microsomal protein and bovine serum albumin. Mechlorethamine interacts with thiol and tyrosyl hydroxyl groups of bovine serum albumin. The use of the mechlorethamine-bovine serum albumin interaction as a model system for the study of the reactions of isophosphamide metabolites with protein is proposed.

Keyphrases ☐ Isophosphamide alkylating metabolites—interaction with microsomal protein and bovine serum albumin, compared to mechlorethamine ☐ Bovine serum albumin—interaction with isophosphamide alkylating metabolites, compared to mechlorethamine ☐ Microsomal protein binding—isophosphamide alkylating metabolites ☐ Mechlorethamine—interaction with microsomal protein and bovine serum albumin, model for isophosphamide interaction

Isophosphamide [3-(2-chloroethyl)-2-(2-chloroethyl)-aminotetrahydro-1,3,2-oxazaphosphorine-2-oxide][1] is an antineoplastic analog of cyclophosphamide currently un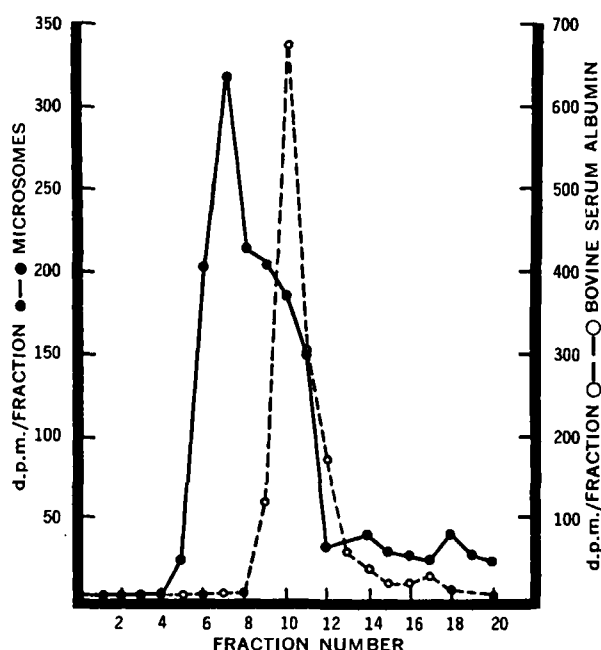dergoing clinical trial. It was previously re-ported (1, 2) that isophosphamide is activated by rat liver microsomes to compounds with alkylating activity. These compounds could react with microsomal protein and so affect the metabolism of isophosphamide itself or of concurrently administered drugs. Indirect evidence for such an interaction is the finding of Sladek (3) that the microsomal metabolism of cyclophosphamide requires fresh microsomes; the process of freezing and thawing even once destroys most of the activity, whereas most other microsomal enzyme activities are relatively resistant to this procedure (4). Similar observations with isophosphamide (2) have been made, and it was postulated that freezing and thawing of microsomes could expose sensitive thiol groups which would be susceptible to alkylation by metabolites of isophosphamide. Therefore, the interaction between the metabolites of isophosphamide and both microsomal protein and bovine serum albumin was examined. As a model of this interaction, the reaction of an alkylating agent of known structure, mechlorethamine, and bovine serum albumin was studied.

MATERIALS AND METHODS

Isophosphamide[2], (U-chloroethyl-^{14}C)-isophosphamide[2], crystalline bovine serum albumin[3], mechlorethamine[3], and dithiobis-nitrobenzoic acid[3] were used. ^{14}C-Isophosphamide (190 μc./μmole), 3.8 mM final concentration, was incubated for 15 min. with a rat liver microsomal suspension (3 ml.) containing microsomes (≅ 250 mg. wet weight liver) (2) in a dialysis bag suspended in 0.5% bovine serum albumin (10 ml.). After incubation the microsomal protein and bovine serum albumin were precipitated with 28% perchloric acid, washed, resuspended, dialyzed against water (10 l.), and subjected to gel filtration on Biogel P-30 to remove noncovalently
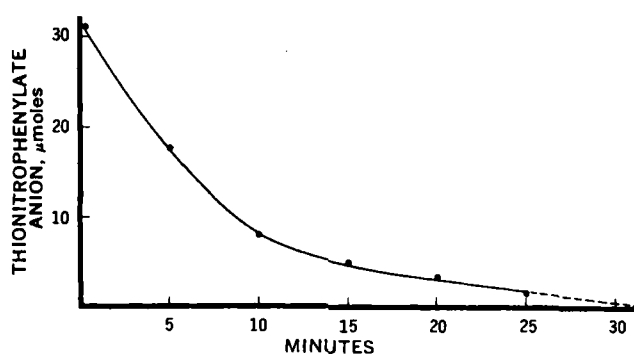

Figure 1—Gel filtration of ^{14}C-isophosphamide-labeled bovine serum albumin (O) and rat liver microsomes (●). Bovine serum albumin and rat liver microsomes were labeled with ^{14}C-isophosphamide as described in the text and were gel filtered through Biogel P-30 in 0.05 M phosphate buffer, pH 7.4; 1-ml. fractions were collected and counted. ^{14}C-Isophosphamide had an elution volume of 40 ml.

Figure 2—Inhibition of the thiol group of bovine serum albumin by mechlorethamine. Bovine serum albumin was treated with mechlorethamine and assayed for free sulfhydryl groups with dithiobisnitrobenzoic acid as described in the text, and the decrease in thionitrophenylate anion with the incubation time of mechlorethamine was plotted.
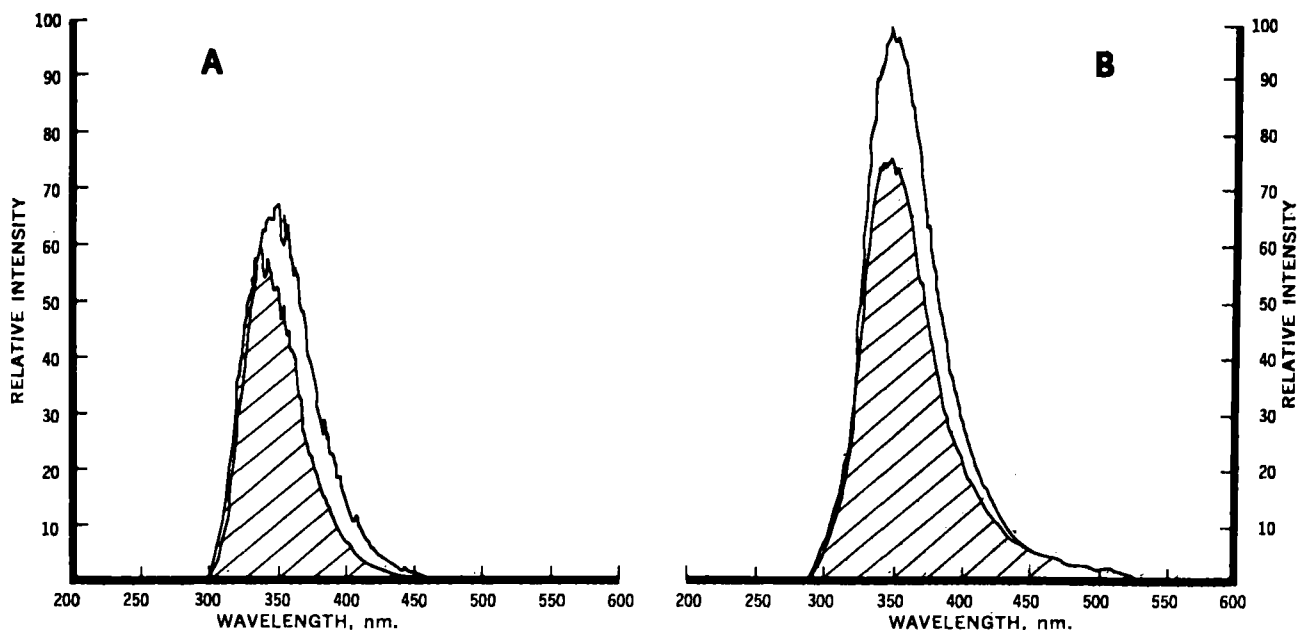
Figure 3—*Fluorescence spectral analysis of bovine serum albumin interaction with mechlorethamine. (A), Fluorescence emission at 287-nm. excitation of bovine serum albumin before (unshaded) and 15 min. after (shaded) addition of mechlorethamine (10.7 μmoles). (B), Fluorescence emission at 305-nm. excitation of bovine serum albumin before (unshaded) and 15 min. after (shaded) addition of mechlorethamine (10.7 μmoles). The slit width was 2.0 mm., sensitivity was 0.001, and photomultiplier was 0.5.*

bound isophosphamide (elution volume 40 ml.). Fractions were counted by liquid scintillation spectroscopy.

The interaction of mechlorethamine with thiol groups and the tyrosine and tryptophan residues of bovine serum albumin was studied by the use of dithiobisnitrobenzoic acid (5) and fluorometry, respectively. Bovine serum albumin (10 mg. in 1.5 ml. of Sorenson's buffer, 0.1 M, pH 7.4) was incubated with mechlorethamine (10.7 μmoles) at 22°. After incubation periods of 0-30 min., dithiobisnitrobenzoic acid (2 mg. in 0.5 ml. of water) was added and the concentration of thiophenylate anion was determined at 412 nm. using an absorptivity of a = 1360.0 (5). Fluorescence spectral analysis of bovine serum albumin (0.22 mg./ml. in 0.067 M phosphate buffer, pH 7.4) was performed on a spectrophotofluorometer[4] at λ_{exc} 287 and λ_{fl} 310 nm. in the presence and absence of mechlorethamine (10.7 μmoles, final concentration).

Ethylmorphine demethylase activity of microsomes was estimated according to Davies et al. (6).

RESULTS

The freezing and thawing procedure of a microsomal preparation greatly increases the inhibition of ethylmorphine demethylase activity by mechlorethamine (Table I). Incubation of isophosphamide with rat liver microsomes leads to covalent binding of isophosphamide metabolites to both microsomal protein and bovine serum albumin (Fig. 1).

A model reaction between mechlorethamine and bovine serum albumin leads to the complete loss of available thiol groups as measured by the dithiobisnitrobenzoic reaction (Fig. 2). Fluorometric determinations indicate no reaction with tryptophan residues [measured by fluorescence at λ_{exc} 305 nm. (6)] but there is a transient decrease in fluorescence intensity at λ_{exc} 287 nm. followed by a 12-nm. shift in λ_{fl} (Fig. 3), indicating an interaction with tyrosine residues (7).

DISCUSSION

The reaction between alkylating agents and nucleophilic centers of proteins is well known (8). Isophosphamide is metabolized into alkylating metabolites by liver microsomes (1, 2), the production

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[4] Aminco-Bowman.

of which occurs in intimate contact with the active sites of microsomal protein and could affect the activity of these enzymes, especially as it is known that microsomal oxidations are inhibited by sulfhydryl reagents (9).

The fact that freezing microsomes greatly reduces their ability to metabolize cyclophosphamide and isophosphamide suggested indirectly that such a reaction could be taking place and that freezing, by exposing essential sulfhydryl groups, was accentuating this effect. The present findings that mechlorethamine inhibits microsomal demethylase activity and that this inhibition is much greater after freezing and thawing the microsomal preparation are in agreement with this concept. The results also show that a reaction does take place between microsomal metabolites of isophosphamide and microsomal protein.

Microsomal protein is heterogeneous and particulate, and the metabolites of isophosphamide are still undefined; consequently, a model for their interaction, consisting of a known alkylating agent and a protein with defined spectral qualities (7) and containing a single thiol group (9), was studied. Interactions between mechlorethamine and both thiol and phenolic residues of bovine serum albumin were demonstrated. A reaction between bovine serum albumin and isophosphamide metabolites was also demonstrated, and it seems to be a convenient, simple model with which to study the interaction between isophosphamide and proteins.

Table I—Effect of Mechlorethamine Pretreatment on Ethylmorphine Demethylase Activity of Rat Liver Microsomes[a]

Mechlorethamine, mM Final Concentration	Ethylmorphine Demethylase Activity, μmoles/g. Liver/hr.			
	Fresh Microsomes	Inhibition, %	Frozen Microsomes	Inhibition, %
0	2.44 ± 0.03	—	2.08 ± 0.00	—
0.5	2.09 ± 0.03	14.3	1.23 ± 0.04	40.9
5.0	0.73 ± 0.03	70.1	0.00 ± 0.00	100

[a] Microsomes prepared as previously described (2) were pretreated with mechlorethamine for 30 min. at 22°. Cofactor mixture (3) and substrate were added and the mixture was incubated for 15 min. at 37° in air with shaking. Each value represents the mean of determinations carried out on three sets of livers, each set being the pooled liver homogenates from two rats.

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Biosynthesis of Deuterated Benzylpenicillins III:
Relative Antibiotic Potency of Highly Deuterated Benzylpenicillin

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Abstract ☐ The relative antibiotic potency of a highly deuterated benzylpenicillin and ^{1}H-benzylpenicillin was compared using the official cup-plate bioassay, with *Sarcina lutea* as the test organism. A relative potency (H/D) of 1.23 was obtained. Although the penicillin analogs act by the same mechanism, deuterium apparently affects the potency in an adverse way.

Keyphrases ☐ Benzylpenicillin, highly deuterated—potency compared to nondeuterated benzylpenicillin, cup-plate bioassay, *Sarcina lutea* ☐ Penicillin analogs—effect of high deuteration on potency of benzylpenicillin ☐ Antibiotics—potency of highly deuterated benzylpenicillin, compared to ^{1}H-benzylpenicillin ☐ Deuteration—effect on potency of benzylpenicillin

The participation of solvent deuterium oxide in the biosynthesis of benzylpenicillin (1) and the isolation and characterization of a highly deuterated benzylpenicillin have been reported (2). Proton magnetic resonance spectra revealed an average replacement of 89% of the protons (^{1}H) with deuterium (^{2}H) atoms. Complete replacement by ^{2}H is observed in the phenylacetyl group, the C-3 position of the thiazolidine ring, and the C-6 position of the β-lactam ring. Partial substitution is noted in the C-5 position of the β-lactam ring (64%) and in the methyl groups (77%) at the C-2 position of the thiazolidine ring.

In the present study, the relative antibiotic potency of this highly deuterated benzylpenicillin and ^{1}H-benzylpenicillin is compared.

EXPERIMENTAL

Highly Deuterated Benzylpenicillin—Isolation, identification, and characterization of a highly deuterated benzylpenicillin was described earlier (2).

Assay—USP XVII (3) describes a relative potency assay which is useful for determining benzylpenicillin activity as compared to a standard. The cup-plate bioassay, involving *Sarcina lutea* (ATCC 9341) as the test organism (1), was used for this study. Penicillin concentrations are expressed in moles rather than in units per milli-
liter or milligrams per milliliter. Expression of the concentrations on a weight basis would introduce a factor involving the difference in molecular weights and would influence the slope in a dose-response relationship (4).

RESULTS AND DISCUSSION

Figure 1 shows the regression lines calculated for highly deuterated potassium benzylpenicillin (D) and potassium ^{1}H-benzylpenicillin (H) when the results of the assay were represented as inhibition zone diameters (millimeters) on the Y-axis (random variable) and the antibiotic concentration was expressed as log moles $\times$ 10^{11} on the X-axis. The statistical methods used by Laskar and Mrtek (4) for their comparisons were utilized here, and a summary of the results is presented in Table I. There was an observed difference in the regression coefficients for the two penicillins, and it was necessary to test the significance. The null hypothesis stated that there was no difference between the slopes of the lines. The results of the slope test were that $t = 0.1625$ (76 df) (not significant), the null hypothesis was accepted, and the parallelism of the slopes was retained. A further test on the regression lines must establish the fact that, although the lines were parallel, intercepts for the two lines were not identical. This test arises from the fact that the intercepts may describe the same locus, and the null hypothesis then was that both regression lines describe the same locus. Application of an identity test reveals that $t = 12.90$ (77 df) ($p < 0.001$) and the null hypothesis can be rejected, indicating that the lines are not identical.

Horizontal displacement may be used to give the relative potency of the ^{1}H- and highly deuterated benzylpenicillins. This calculation revealed a relative potency (H/D) of 1.23 (95% confidence interval, 1.20–1.26) and indicated that, although the analogs presumably work by the same mechanism of action, deuterium in the molecule affected the potency adversely. Laskar and Mrtek (4) found a relative potency of 1.25 (H/D) for deuteriobenzyl-d_7-penicillin; but a comparison, although tempting, between the results of these workers and the present study would be only cursory since two methods of assay (turbidimetric and cup plate, respectively), two organisms (*Staphylococcus aureus* and *S. lutea*, respectively), and two salts (*N*-ethylpiperidine and potassium, respectively) were used.

Interpretation of Assay Results—A review of the effects of deuterium on therapeutic compounds was presented by Katz and Crespi (5). Of particular interest are the effects of deuterium substitution on the activity of other antibiotic compounds. Nona *et al.* (6) isolated ^{2}H-griseofulvin and evaluated its *in vitro* activity. The fully deuterated analog was found to be slightly more potent than