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AN UNEXPECTED PATHWAY FOR THE METABOLIC DEGRADATION OF 1,3-DIALKYL-3-ACYLTRIAZENES

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Abstract—In the presence of NADPH, rat liver microsomes catalyzed the degradation of a series of 1,3-dialkyl-3-acyltriazenes, and the extent of the reaction was correlated with compound lipophilicity. In the case of two methylcarbamoyltriazenes, 1-(2-chloroethyl)-3-benzyl-3-(methylcarbamoyl)triazene (CBzM) and 1-(2-chloroethyl)-3-methyl-3-(methylcarbamoyl)triazene (CMM), microsomal metabolites were isolated. Identification of the CBzM metabolites as 1-(2-chloroethyl)-3-benzyl-3-(hydroxymethylcarbamoyl)triazene and 1-(2-chloroethyl)-3-benzyl-3-carbamoyltriazene, and the CMM metabolite as 1-(2-chloroethyl)-3-methyl-3-(hydroxymethylcarbamoyl)triazene indicated that the first metabolic step involves hydroxylation of the methylcarbamoyl substituent. Detailed studies of the metabolism of CBzM indicated that the K_m for the reaction was 84 μM, and that metabolism was more efficient if microsomes were prepared from male than from female rats. During prolonged incubation, the metabolites of CBzM were also degraded. The degradation of CBzM and its metabolites was inhibited by SKF-525A and metyrapone, suggesting the involvement of a cytochrome P450 isozyme, and supporting the hypothesis that the process is oxidative rather than hydrolytic in both cases. Metabolic oxidation represents an alternative pathway to chemical or enzymatic hydrolysis for the *in vivo* decomposition of (methylcarbamoyl)triazenes. This mechanism may ultimately explain the antitumor efficacy and low acute toxicity of selected compounds.

Arylalkyltriazenes have been a subject of study by chemists and biologists for many years. Interest in these compounds is attributable, at least in part, to the fact that some possess antitumor activity, and one, 5(4)-(3,3-dimethyl-1-triazeno)imidazole-4(5)-carboxamide (DTIC§) has proven to be therapeutically valuable. The basis for the biological action of the arylalkyltriazenes lies in their capacity to alkylate DNA, often subsequent to a metabolic activation step. In the case of DTIC, hydroxylation of one of the triazene methyl groups is followed by spontaneous demethylation. The resulting unstable 5(4)-3-(methyl-1-triazeno)imidazole-4(5)-carboxamide then undergoes chemical decomposition to yield 4-aminoimidazole-5-carboxamide and methyldiazohydroxide. The methyldiazohydroxide further decomposes to form the methyldiazonium ion, an extremely reactive DNA methylating agent [1].

Work in our laboratories has focused on the chemically similar 1,3-dialkyltriazenes and 1,3,3-trialkyltriazenes [2–7]. These compounds are also DNA-alkylating agents due to the fact that their rapid, proteolytic decomposition yields the alkyldiazonium ion [8]. In this case, however, there is no need for metabolic activation, since the di- and trialkyltriazenes are highly unstable. Not surprisingly, selected alkyltriazenes have been shown to be both carcinogenic and mutagenic in a number of biological systems [9, 10].

Although alkyltriazenes have been useful model compounds for the study of DNA alkylation in the absence of metabolic activation, their extreme chemical instability has rendered more complex biological studies difficult [4, 5]. Therefore, in an effort to acquire greater chemical stability while retaining alkylating activity, the 1,3-dialkyl-3-acyltriazenes‖ were developed [11]. The general structure and specific substituents for these compounds are given in Table 1. The 1,3-dialkyl-3-acyltriazenes also decompose chemically to yield alkyldiazonium ions, but via a more complex mechanism. In most cases, they exhibit acid-catalyzed decomposition at lower pH, uncatalyzed decomposition at neutral pH, and base-catalyzed decomposition at higher pH, with the specific pH ranges dependent upon the substituents of

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§ Abbreviations: DTIC, 5(4)-(3,3-dimethyl-1-triazeno)-imidazole-4(5)-carboxamide; CBzM, 1-(2-chloroethyl)-3-benzyl-3-(methylcarbamoyl)triazene; CMM, 1-(2-chloroethyl)-3-methyl-3-(methylcarbamoyl)triazene; CMC, 1-(2-chloroethyl)-3-methyl-3-carbethoxytriazene; CMA, 1-(2-chloroethyl)-3-methyl-3-acetyltriazene; HBzM, 1-(2-hydroxyethyl)-3-benzyl-3-(methylcarbamoyl)triazene; HMM, 1-(2-hydroxyethyl)-3-methyl-3-(methylcarbamoyl)-triazene; and HMA, 1-(2-hydroxyethyl)-3-methyl-3-acetyltriazene.

‖ The term 1,3-dialkyl-3-acyltriazenes here refers to compounds in which nitrogen(3) is directly attached to a carbonyl-containing substituent. This generic term is used for convenience to emphasize the common chemical behavior resulting from the presence of the carbonyl moiety, although it is noted that the carbethoxy and methylcarbamoyl substituents are not acyl groups in the strictest usage of the term.

the compound [12, 13]. The acid-catalyzed and -uncatalyzed processes involve heterolytic cleavage of the N(2)—N(3) bond, yielding an alkyldiazonium ion from the N(1) substituent. It is expected that this hydrolytic process should prevail under physiological conditions.

Based on these assumptions, a series of 1,3-dimethyl-3-acyltriazenes and of 1-(2-chloroethyl)-3-methyl-3-acyltriazenes were synthesized as sources of the methyldiazonium ion and the (2-chloroethyl)-diazonium ion, respectively [14, 15]. *In vitro* DNA alkylation by four of these compounds at pH 7.5 was totally consistent with expectations based on N(2)—N(3) heterolysis, resulting in methylation in the case of compounds bearing a 1-methyl substituent, and chloroethylation or hydroxyethylation in the case of compounds bearing a 1-(2-chloroethyl) substituent [16]. However, the *in vitro* studies raised an important question concerning the ability of these compounds to alkylate DNA *in vivo*. In particular, their high degree of chemical stability under physiological conditions dictated incubation periods of 24 hr or more in some cases, leading us to ask if breakdown to the diazonium ion would occur prior to metabolism or excretion. A partial answer to this question came from further studies of DNA alkylation by 1-(2-chloroethyl)-3-methyl-3-carbethoxytriazene (CMC). In the presence of porcine liver esterase, the rate and extent of alkylation by this compound were increased markedly. This was due to the fact that the esterase catalyzed removal of the carbethoxy moiety, yielding the highly unstable dialkyltriazene which rapidly decomposed to form the alkyl-diazonium ion. Thus, it was postulated that CMC and similar compounds would be potent alkylating agents *in vivo*, but via a prior enzymatic deacylation step [16].

Biological studies of (2-chloroethyl)alkylacyl-triazenes and dimethylacyltriazenes revealed that they possessed cytotoxic activity in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) microculture tetrazolium assay, with the (2-chloroethyl)alkyltriazenes generally showing higher potency than dimethyltriazines [11]. These compounds were also tested against several tumor xenografts in nude mice. Results showed that CMC was highly toxic, but that another compound, 1-(2-chloroethyl)-3-methyl-3-(methylcarbamoyl)triazene (CMM), not only effected cures in the cases of the P388 murine leukemia and the LOX melanoma, but also showed little acute toxicity even at the relatively high doses of 800 mg/kg [11]. CMC and CMM differ only in the acyl substituent, and their chemical behavior at physiological pH is highly similar, although CMM is more stable to proteolytic decomposition. Thus, the great disparity in their toxicity led to the hypothesis that their behavior *in vivo* must be strikingly different. Consequently, we have conducted studies on the metabolism of (methylcarbamoyl)triazenes, and herein present evidence that the (methylcarbamoyl)triazenes are subject to oxidative degradation as opposed to deacylation. Furthermore, the oxidative pathway likely does not yield an alkyldiazonium ion, or similar highly reactive alkylating species. Therefore, these compounds may represent a class of antitumor triazenes that function via a unique mechanism that is associated with markedly reduced toxicity.

MATERIALS AND METHODS

Animals. Young adult male Fischer 344 rats (8–10 weeks) from the colony of the Frederick Cancer Research and Development Center were maintained on standard laboratory diet and given water *ad lib.*

Chemicals. Triazenes were prepared, purified, and characterized according to the procedure of Smith *et al.* [14, 15], and were >99% pure as determined by NMR and UV/vis spectroscopy, and HPLC. Triazene stock solutions were 100 or 300 mM in acetonitrile, and were stored at −20°. Previous experience has shown that these solutions are stable for months, even at room temperature, provided they are protected from light.

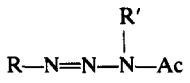
NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, SKF-525A, and metyrapone were purchased from the Sigma Chemical Co. (St. Louis, MO).

Rat liver microsomes and other subcellular fractions. Rats were killed by CO_2 asphyxia. Livers were immediately removed, and washed in a solution of 0.025 M Tris–HCl, 0.001 M ethylenediaminetetraacetic acid, 0.34 M sucrose, pH 7.5. The livers were then homogenized in the same buffer (3 mL/g tissue) by four passes in a Potter–Elvehjem homogenizer. Following centrifugation at 9,000 *g* for 15 min, the supernatants were subjected to further centrifugation at 100,000 *g* for 60 min. The resulting pellets (9,000 *g* and 100,000 *g*) were suspended in one-third the original homogenate volume of buffer. The 100,000 *g* pellets (microsomes) contained approximately 40 mg/mL protein as determined by the method of Bradford [17]. For storage, the suspensions were made 20% in glycerol and maintained at −20° (short term) or −80° (long term). The activity was stable for up to 6 months under the long-term storage conditions.

Incubations of triazenes with rat liver microsomes. Incubation mixtures contained 0.1 M Tris–HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 1 mM NADP, 5 mM glucose-6-phosphate, 0.2 U/mL glucose-6-phosphate dehydrogenase, 3 mg microsomal protein/mL, and the desired final concentration of triazene added as a stock solution in acetonitrile. In general, all components of the reaction mixture were combined except for the microsomes. Samples were then warmed to 37°, and the microsomes were added to initiate the reaction. Aliquots (100 μL) were removed at desired time points for triazene assay.

Assay of triazene metabolism. The disappearance of triazenes and the appearance of triazene metabolites were monitored by HPLC. Aliquots of 100 μL were removed from incubation mixtures and combined with 100 μL of methanol containing the appropriate triazene internal standard on ice. Following the addition of 100 μL of saturated barium hydroxide and 100 μL of 5% (w/v) zinc sulfate, precipitates were removed by centrifugation. The resulting supernatants were analyzed by reverse-phase HPLC on a column (4.6 × 250 mm) of Microsorb C18 (Rainin Instrument Co.) eluted at a flow rate of 1 mL/min with a mixture of methanol–

Table 1. Structures of acyltriazenes and conditions for HPLC assay



Abbreviation	R	R′	Ac	Internal standard	Eluent*	Retention time (min)	$T_{1/2}$† (min)
CBzM	2-Chloroethyl	Benzyl	Methylcarbamoyl	CMM	60:40	12.0	279
CMM	2-Chloroethyl	Methyl	Methylcarbamoyl	CMA	50:50	6.2	470
CMA	2-Chloroethyl	Methyl	Acetyl	CMM	50:50	9.7	259
HBzM	2-Hydroxyethyl	Benzyl	Methylcarbamoyl	HMM	45:55	10.5	22.4
HMM	2-Hydroxyethyl	Methyl	Methylcarbamoyl	HMA	25:75	5.5	53.5
HMA	2-Hydroxyethyl	Methyl	Acetyl	HMM	25:75	7.6	25.5
CMC	2-Chloroethyl	Methyl	Carbethoxy	NA	NA	NA	NA

* Eluent concentrations are given as the ratio of methanol:water (v/v).
† Half-life for chemical decomposition at pH 7.5 and 70° [11].

water of appropriate composition. The effluent was monitored for absorbance at 245 nm, and triazenes were quantitated on the basis of the peak area relative to the peak area of the internal standard. The relative peak area obtained from samples taken at the beginning of the incubation was assumed to represent the known concentration of triazene added to the incubation mixture. From this relationship, absolute triazene concentrations were calculated for samples from later time points. This method was also used for triazene metabolites, under the assumption that the detector response for these compounds would be similar to that of the parent triazene. Table 1 summarizes HPLC conditions for each of the triazenes, including internal standard, eluent composition, and retention time.

Isolation of triazene metabolites. Triazene incubation mixtures (20 mL) containing 300 μM triazene were incubated for 3 hr. The reactions were stopped by the addition of 10 mL of saturated barium hydroxide and 10 mL of 5% (w/v) zinc sulfate. The precipitate was removed by filtration, and the filtrate was extracted twice with 40 mL of methylene chloride. The extracts were combined, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. Residues were dissolved in 0.4 mL of methanol:water (50:50, v/v) and purified by HPLC in the appropriate solvent system.

Analyses. NMR spectra were obtained on a Varian VXRS 500 MHz spectrometer equipped with a SUN 4/110 data station.

Mass spectra were obtained on a VG 70–250 mass spectrometer (VG Analytical). The mass spectrometer was operated in the positive ion detection mode. Ion formation was by chemical ionization performed at an emission current of 0.5 mA and an electron energy of 50 eV. The ion source temperature was 180°. Anhydrous ammonia was used as the reagent gas at a pressure of 5×10^{-5} millibar. The instrument was scanned from m/z 650 to m/z 40 at 3 sec/decade with a 0.5-sec magnet reset time and a resolving power of 3000. The accelerating voltage was 6000 V. Accurate masses were examined by peak matching using the mass spectrometer data system and perfluorokerosene as the source of reference peaks. Samples were introduced via the solid probe.

HPLC was performed on an instrument composed of an SCL-6B system controller driving two LC-6A pumps, a SPD6AV UV/vis spectrophotometric detector and a CR601 chromatopac data integrator, all obtained from Shimadzu Scientific Instruments.

RESULTS

Comparison of metabolism of acyltriazenes. In early studies, we incubated a series of six acyltriazenes (Table 1) with rat liver microsomes, as described in Materials and Methods. All triazenes were present initially at concentrations of 120 μM, and the incubations were carried out for 3 hr. Control incubations were identical except that the microsomes were omitted. Aliquots were taken at the beginning and end of the incubation period for assay of triazene concentration. Figure 1 summarizes the results of these experiments. In the absence of microsomes, only 1-(2-hydroxyethyl)-3-methyl-3-acetyltriazene (HMA) and 1-(2-hydroxyethyl)-3-benzyl-3-(methylcarbamoyl) triazene (HBzM) showed an obvious decrease in concentration during the 3-hr incubation. The decrease was statistically significant only for HMA. This is consistent with the fact that these two tiazenes have the shortest half-life for chemical decomposition of the ones tested (Table 1). In contrast, the microsomes catalyzed the disappearance of four of the six triazenes, specifically CMM, HBzM, 1-(2-chloroethyl)-3-methyl-3-acetyltriazene (CMA), and 1-(2-chloroethyl)-3-benzyl-3-(methylcarbamoyl)triazene (CBzM), listed in order of increasing breakdown. It is interesting to note that the extent of degradation of these triazenes in the presence of microsomes was directly related to their retention time on reverse-phase HPLC. Thus, when the eluent was methanol:water (50:50, v/v), the retention times for 1-(2-hydroxyethyl)-3-methyl-3-(methylcarbamoyl)triazene (HMM), HMA, CMM, HBzM, CMA, and CBzM were 2.9, 3.4, 6.2, 7.5, 9.7, and 31.1 min, respectively.

Liver microsomes—the major source of CBzM-metabolizing enzymes. The initial experiments

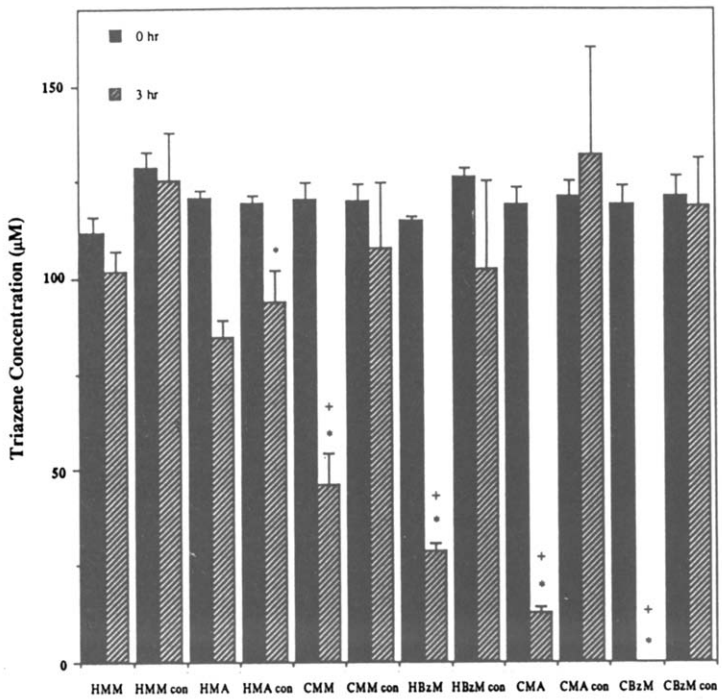


Fig. 1. Comparison of the microsomal metabolism of acyltriazenes. The six indicated acyltriazenes were incubated with or without (con) microsomes for 3 hr as described in Materials and Methods. Initial concentrations of all triazenes were 120 μM. Samples for assay of triazene concentration were taken in triplicate at the beginning and end of the incubation period. The results presented are the means ± SD of three separate experiments. (Key) (*) values at 3 hr were significantly different from 0 hr ($P < 0.05$), and (+) values at 3 hr were significantly different from the control values at 3 hr ($P < 0.05$).

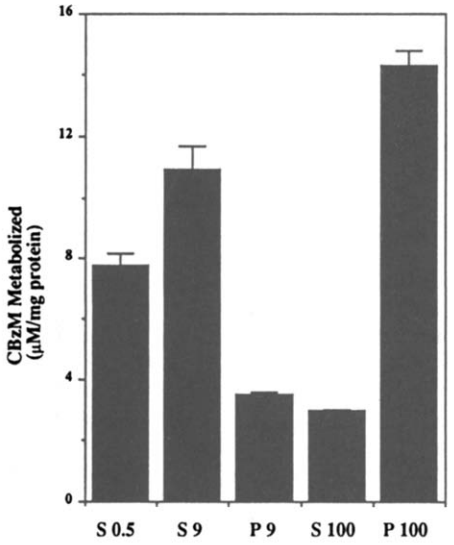


Fig. 2. Capacity of subcellular fractions from rat liver homogenate to metabolize CBzM. Subcellular fractions were prepared from rat liver homogenate as described in Materials and Methods. Each fraction was then incubated with CBzM (initial concentration 100 μM) for 3 hr. Data represent the quantity of CBzM metabolized per milligram fraction protein and are the means ± SD of triplicate determinations for the homogenate (500 g supernatant, S 0.5), the 9,000 g supernatant (S 9), the 9,000 g pellet (P 9), the 100,000 g supernatant (S 100) and the 100,000 g pellet (P 100).

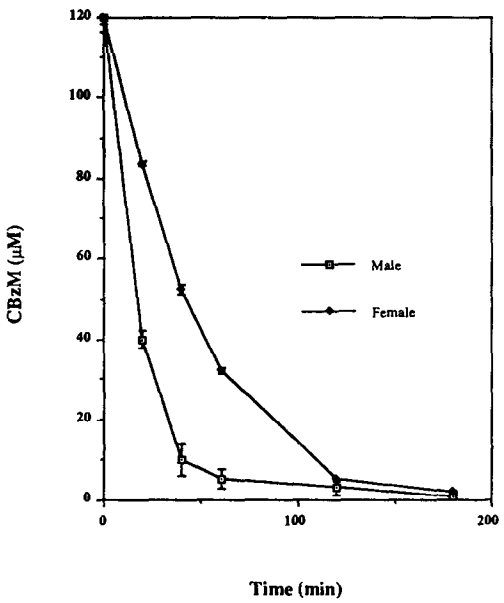


Fig. 3. Rate of CBzM metabolism by microsomes from livers of male and female rats. Rat liver microsomes were prepared from male and female rats as described in Materials and Methods. The microsomal preparations were then incubated with CBzM (initial concentration 120 μM) for the indicated times, and samples were removed for assay of CBzM concentration. Data are the means ± range of duplicate determinations.

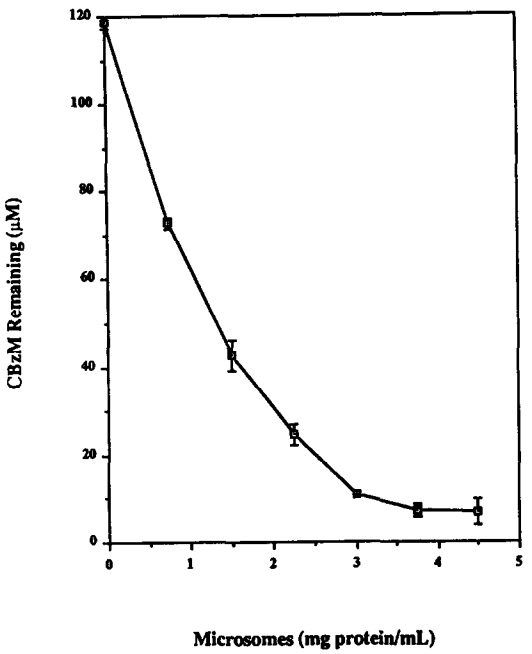


Fig. 4. Effect of microsomal concentration on extent of CBzM metabolism. CBzM was incubated with microsomes for 25 min as described in Materials and Methods, except that the quantity of microsomal protein was varied as indicated. The initial concentration of CBzM was 120 μM. Triplicate samples were taken at the beginning and end of the incubation period. Results are the means ± SD of triplicate determinations.

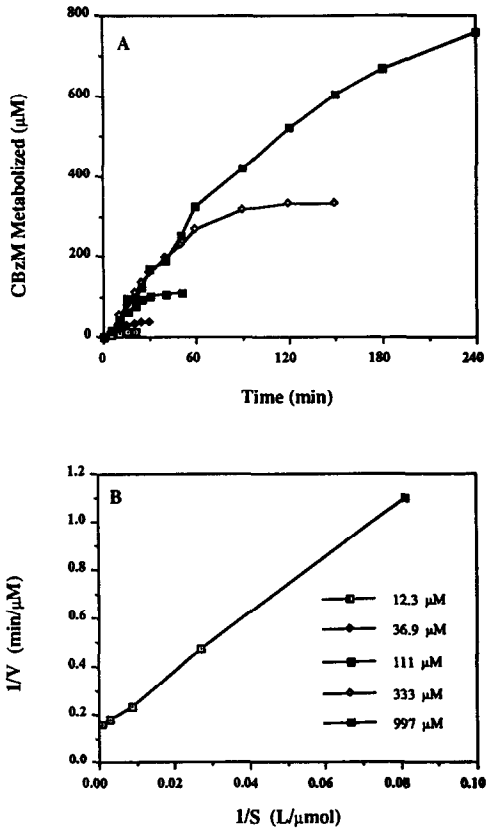


Fig. 5. Effect of CBzM concentration on rate of metabolism. (A) CBzM was incubated with microsomes as described in Materials and Methods, with initial concentrations varied as indicated. Duplicate samples were removed for assay at the designated time points, and the quantity of CBzM remaining was measured. The amount of CBzM metabolized was then determined by subtracting the remaining quantity from the initial concentration. Results are the means of three separate experiments. (B) Data from A were used to calculate initial rates of metabolism at the various CBzM concentrations, and the results were used to construct a Lineweaver–Burk plot.

demonstrated that rat liver microsomes could catalyze the breakdown of certain acyltriazenes. However, they provided no information as to the nature of the reactions involved. As CBzM was the most extensively metabolized of all the triazenes tested, it was selected for further, detailed studies. It was first desirable to determine whether liver microsomes were the only source of enzymes that could metabolize this compound. Differential centrifugation of a rat liver homogenate indicated that little metabolizing activity was associated with other cellular fractions from this tissue (Fig. 2). In addition, a comparison of 9000 *g* supernatants from liver, lung, and kidney prepared in the same way demonstrated that the liver supernatant metabolized 99% of CBzM originally present at a 120 μM concentration, whereas the lung and kidney supernatants each metabolized less than 1% under identical conditions. Of additional interest were our findings that liver microsomes from male rats were more efficient sources of CBzM-metabolizing activity than similarly prepared microsomes from female rats (Fig. 3). Together these data support the hypothesis that the major source of enzymes for the metabolism of CBzM resides in liver microsomes, and that the particular enzyme(s) involved may be under hormonal control.

Kinetics of the microsomal metabolism of CBzM. Figure 4 shows the results of experiments in which 120 μM CBzM was incubated for 25 min with various concentrations of microsomes. As expected, the extent of metabolism was directly related to microsomal concentration. The relationship appears linear only at the lower concentrations of microsomes, due to the fact that over 80% of the substrate was consumed at the higher concentrations. Figure 5A shows the results of experiments in which the rate of CBzM metabolism was monitored at different substrate concentrations. Initial rates estimated from the early time points of these data yielded the Lineweaver–Burk plot shown in Fig. 5B. Linear regression analysis produced an estimate of 84 μM for K_m and 7.1 μM/min for V_{max}. These values should be viewed with caution, however, considering the nature of the discontinuous assay used to obtain them, and the fact that the enzyme system was impure.

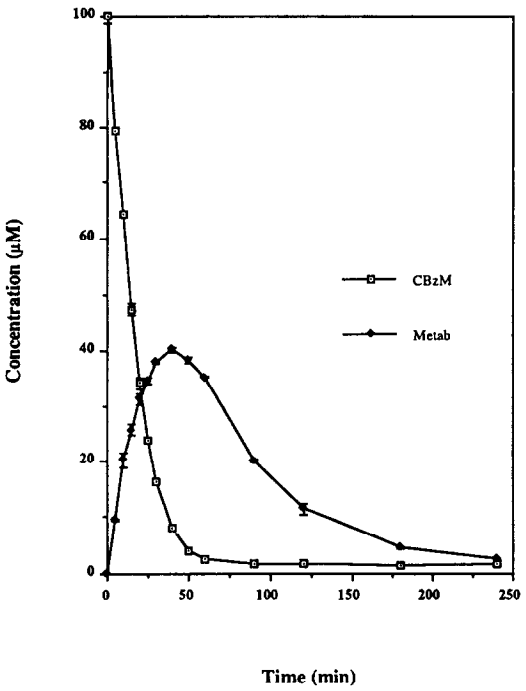


Fig. 6. Time course of the formation and degradation of CBzM metabolites. CBzM (initial concentration 100 μM) was incubated with microsomes as described in Materials and Methods, and duplicate samples were removed at the indicated time points for assay of CBzM and metabolite concentrations. Each data point shows the mean ± range for duplicate determinations for CBzM and the combined metabolites.

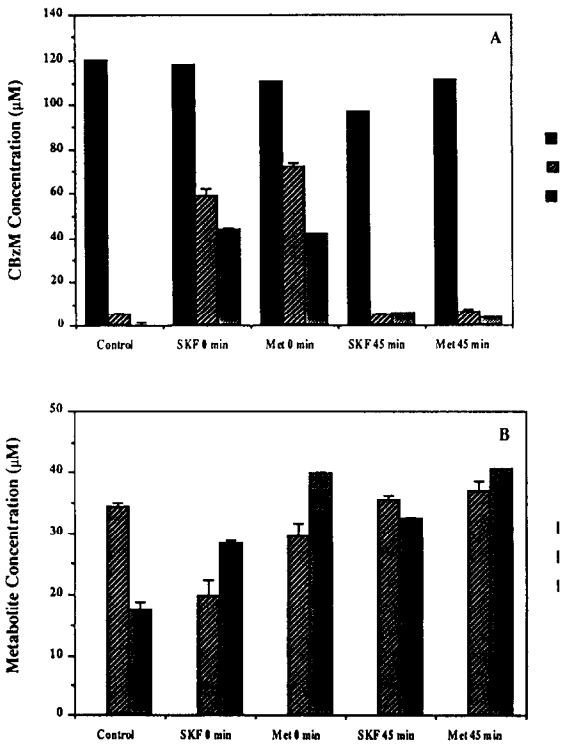


Fig. 7. Effects of cytochrome P450 inhibitors on CBzM metabolism. CBzM (initial concentration 120 μM) was incubated with microsomes as described in Materials and Methods, and duplicate samples were removed initially (0 min), and after 45 min and 120 min of incubation for assay of CBzM and its metabolites. In control samples, no inhibitor was present. In samples labeled SKF 0 min, samples contained SKF-525A (50 μM) throughout the incubation. In samples labeled Met 0 min, samples contained metyrapone (1 mM) throughout the incubation. In samples labeled SKF 45 min, 50 μM SKF-525A was added after 45 min of incubation. In samples labeled Met 45 min, 1 mM metyrapone was added after 45 min of incubation. Data are the means ± range of duplicate determinations for the quantity of CBzM (A) and the combined metabolites (B).

Appearance of metabolites during the metabolism of CBzM. During the time–course studies of CBzM metabolism, it was noted that two new peaks appeared on HPLC chromatograms as the peak for CBzM disappeared. These peaks had retention times of 7.8 and 8.6 min when the eluent was methanol:water (60:40, v/v). Their relative proportions varied among different experiments, although the first peak (retention time = 7.8 min) was usually the larger of the two. With prolonged incubation, these peaks disappeared, suggesting that they were also being metabolized. There was no clear evidence that one of the peaks gave rise to the other. Figure 6 shows the time course of the metabolism of CBzM, including the appearance and disappearance of the metabolites.

Probable involvement of cytochrome P450 in CBzM metabolism. Initial studies had indicated that metabolism of CBzM did not occur when the NADPH-generating system was omitted from the incubation mixture. This observation suggested that the metabolic process involved oxidation of the compound, rather than hydrolytic deacylation. To further test this hypothesis, we performed an experiment in which the effects of two broad spectrum inhibitors of cytochrome P450 isozymes, metyrapone and SKF-525A, were examined [18]. In this experiment, CBzM was incubated with liver microsomes, and samples were removed from incubation mixtures at 0, 45 and 120 min for assay of CBzM and its metabolites. Incubations were carried out in the absence of inhibitor, in the presence of each inhibitor for the full time period, or in the presence of each inhibitor only after the first 45 min of the incubation period. The results are shown in Fig. 7. As seen in Fig. 7A, CBzM was metabolized almost completely by 45 min when no inhibitors were present. Inclusion of inhibitors throughout the incubation period resulted in an approximately 40% inhibition of CBzM metabolism over the 2-hr period. When inhibitors were added after 45 min, the metabolism of the small amount of remaining CBzM was also inhibited. As seen in Fig. 7B, when no inhibitors were present, the concentration of metabolites were higher at 45 min than at 120 min, suggesting that metabolism of the

metabolites was taking place. When inhibitors were present throughout the incubation period, less metabolite was present at 45 min than in control incubations, due to the slower rate of CBzM metabolism. However, metabolite levels continued to increase between 45 and 120 min in these samples, due to the fact that CBzM was still present, and its metabolism was continuing. When inhibitors were added after 45 min of incubation, one sees that the metabolism of the metabolites was blocked, with no decrease in levels between the 45- and 120-min time points. This could not likely be due to the production of additional metabolite, since the concentration of CBzM was so low in these samples after 45 min. These results are consistent with the hypothesis that one or more of the isozymes of cytochrome P450 is involved in the metabolism of CBzM and its metabolite, and that both of these processes are oxidative rather than hydrolytic.

Isolation of the metabolites of CBzM. To better determine the mechanism of CBzM metabolism, the metabolites were isolated by methylene chloride extraction and HPLC from a mixture in which a 300-μM initial concentration of CBzM had been incubated for 3 hr with microsomes. To separate the two metabolites, elution was carried out with methanol:water (50:50, v/v), and the compounds eluted at 17.2 min (CBzM-met1) and 19.6 min (CBzM-met2). ^{1}H NMR spectroscopy provided the following data for CBzM-met1: (CD_3CN, Me_4Si) δ 3.73 (1H, t, J = 6.9 Hz), 3.87 (2H, t, J = 5.7 Hz), 4.03 (2H, t, J = 5.7 Hz), 4.76 (2H, d of d, J = 6.5, 6.7 Hz), 5.09 (2H, s), 7.24 (5H, m), 7.61 (1H, broad). After D_2O exchange, signals at 3.73 and 7.61 ppm disappeared, and the signal at 4.76 became a singlet. COSY analysis indicated coupling between the signals at 3.87 and 4.03 ppm, as well as between signals at 3.73 and 4.76 ppm and between signals at 4.76 and 7.61 ppm. For CBzM-met2, ^{1}H NMR revealed: (CD_3CN, Me_4Si) δ 3.86 (2H, t, J =5.8 Hz), 4.01 (2H, t, J = 5.7 Hz), 5.06 (2H, s), 5.6 (1H, broad), 6.5 (1H, broad), 7.23 (5H, m). Comparison of these spectra to the previously published spectrum of CBzM ($CDCl_3$, Me_4Si) δ 2.97 (3H, d, J = 4.8 Hz), 3.77 (2H, t, J = 5.7 Hz), 3.98 (2H, t, J = 5.7 Hz), 5.14 (2H, s), 6.34 (1H, broad), 7.25 (5M, m) [12], has led to the identification of CBzM-met1 as 1-(2-chloroethyl)-3-benzyl-3-(hydroxymethylcarbamoyl) triazene, and CBzM-met2 as 1-(2-chloroethyl)-3-benzyl-3-carbamoyltriazene. The structures were supported by chemical ionization mass spectroscopy that yielded the correct mass of 271.0937 amu (calculated 271.0961 amu for molecular formula $C_{11}H_{15}N_4O_2Cl$-H^+) for CBzM-met1 and 241.0880 amu (calculated 241.0856 amu for molecular formula $C_{10}H_{13}N_4OCl$-H^+) for CBzM-met2.

Rate of metabolism of CMM, and formation of a CMM metabolite. CBzM had proven to be a useful model system for the study of (methylcarbamoyl)triazene metabolism. However, because no data are presently available concerning the biological effectiveness of CBzM, it was desirable to also study CMM, for which such data are available. Figure 8 shows the rate of disappearance of CMM from an incubation mixture in which a 100 μM concentration of the compound was incubated with rat liver microsomes. The degradation of the triazene required the presence of both microsomes and NADPH (data not shown). As expected from earlier experiments (Fig. 1), CMM was metabolized more slowly and less completely than CBzM under the same conditions. During the course of the incubation, however, a new peak appeared having an HPLC retention time of 4.4 min in methanol:water (50:50, v/v). Unlike the case for CBzM, only one peak was usually observed; however, this peak often had a shoulder at 4.7 min. Neither CMM nor the metabolite peak completely disappeared, even after prolonged incubation (up to 10 hr).

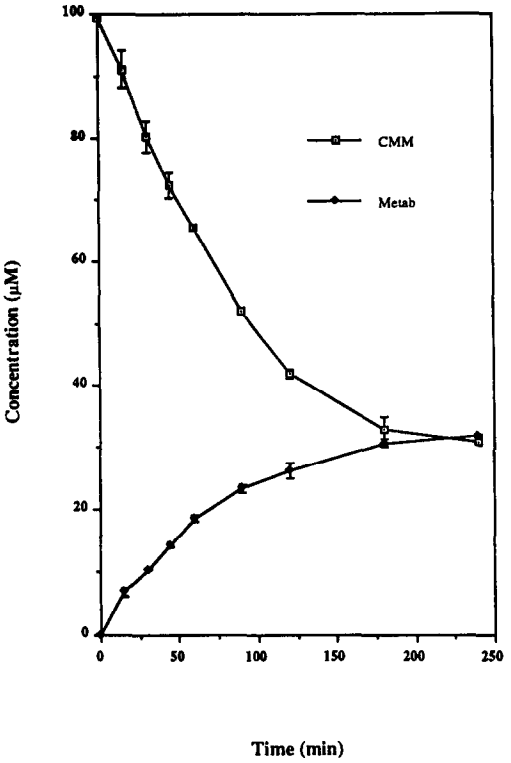


Fig. 8. Time course of the degradation of CMM and the formation of the CMM metabolite. CMM (initial concentration 100 μM) was incubated with microsomes as described in Materials and Methods, and duplicate samples were removed at the indicated time points for assay of CMM and its metabolite concentrations. Each data point shows the mean ± range for duplicate determinations.

Isolation of the metabolite of CMM. To identify the structure of the CMM metabolite, this compound was isolated from an incubation mixture in which 300 μM CMM was incubated with microsomes for 3 hr. Purification of the compound was accomplished by methylene chloride extraction followed by HPLC eluted with methanol:water (30:70, v/v). The compound (CMM-met1) eluted at 10.7 min. ^{1}H NMR revealed: (CD_3CN, Me_3Si) δ 3.18 (3H, s), 3.65 (1H, t, J = 6.6 Hz), 3.94 (2 H, t, J = 5.9 Hz), 4.07 (2H, t, J = 5.7 Hz), 4.73 (2H, d of d, J = 6.4, 6.5 Hz), 7.53 (1H, broad). After D_2O exchange,

signals at 3.65 and 7.53 ppm disappeared, and the signal at 4.73 became a singlet. COSY analysis indicated coupling between the signals at 3.94 and 4.07 ppm, as well as between signals at 3.65 and 4.73 ppm and between signals at 4.73 and 7.53 ppm. Comparison with the previously published NMR of CMM [15] ($CDCl_3$, Me_4Si) δ 2.95 (3H, d, J = 5 Hz), 3.26 (3H, s), 3.86 (2H, t, J = 6 Hz), 4.05 (2H, t, J = 6 Hz), 6.37 (1H, broad), led to the identification of the metabolite as 1-(2-chloroethyl)-3-methyl-3-(hydroxymethylcarbamoyl)triazene. Chemical ionization mass spectroscopy yielded a mass of 195.0638 amu (calculated 195.0648 amu for molecular formula $C_5H_{11}N_4O_2Cl$-H^+), in support of the proposed structure.

DISCUSSION

Initial studies of triazene metabolism demonstrated that incubation of the compounds with rat liver microsomes resulted in a rate of breakdown that was not explained by their chemical stability, indicating the occurrence of an enzymatic process. The finding that the rate of decomposition in the presence of microsomes was correlated with reverse-phase HPLC retention time suggests that the suitability of the triazenes to serve as substrates for this process is determined mainly by their lipophilicity. This is consistent with the involvement of a membrane-associated enzyme, but contrasts with earlier studies of aryldialkyltriazenes that showed metabolism to be inversely correlated with lipophilicity [19]. This discrepancy between the two classes of triazenes may be due to greater steric hinderance in the case of the more lipophilic and bulkier aryldialkyltriazenes that limits their access to the active site of the enzyme(s) [19].

For more extensive metabolic studies, CBzM was chosen, because it is rapidly and extensively metabolized, allowing kinetic and structural data to be obtained with relative ease. Our results enable us to predict that, at least in the rat, CBzM metabolism would occur predominantly in the liver via a microsomal enzyme system, and that the metabolism would be oxidative rather than hydrolytic. The location of the enzyme(s), the requirement for NADPH, and the susceptibility of the enzymatic activity to SKF-525A and metyrapone led us to conclude that one or more isozymes of cytochrome P450 are most likely responsible for the metabolic process. The fact that microsomes from male rats degraded CBzM more rapidly than did those from female rats suggests that the relevant isozyme(s) may be under hormonal control. For example the testosterone-dependent cytochrome P450 isozyme UT-A described by Guengerich *et al.* [20] could be responsible for the enhanced metabolism in male rats.

In the case of both CBzM and CMM, we have isolated a metabolite that appears to have resulted from hydroxylation of the methylcarbamoyl substituent. This is supported by the NMR studies of both metabolites, which indicate that the chloroethyl and benzyl (CBzM) or triazene methyl (CMM) signals are present, but that the methyl signal of the methylcarbamoyl group has disappeared. In both metabolites, this signal has been replaced by a set of three signals at approximately 3.6, 4.7 and 7.5 ppm. These are consistent with the hydroxyl proton, the methylene protons, and the amide proton, respectively, of a hydroxylated methylcarbamoyl group. Unlike compounds such as *N*-nitrosodialkylamines, which undergo rapid N-dealkylation after metabolic hydroxylation of the alkyl moiety [21], hydroxylated (methylcarbamoyl)triazenes are remarkably stable. This is indicated by the fact that these compounds could be isolated with ease by HPLC in protic solvents, a process that often required a period of several days. The unexpected stability of the hydroxymethylcarbamoyl group may be explained by the similarity of the structure to a substituted area. Derivatives of hydroxymethylurea have been well studied, and are sufficiently stable to allow purification of liquid chromatography in protic media [22]. Interestingly, a series of *N*-hydroxymethyl analogues of certain aryldialkyltriazenes have been synthesized, and found to be surprisingly stable, despite the fact that demethylation is thought to be critical for metabolic activation of these compounds [23].

In the case of CBzM, we have isolated a second metabolite identified as the demethylated compound. This suggests that at least some demethylation of CBzM-met1 does occur. In this case, one would expect a time-dependent transition from CBzM-met1 to CBzM-met2 during the course of the CBzM incubation. We did not observe this. However, one must remember that both compounds are further degraded by the microsomal system, a process that may very well obscure the temporal relationship between the two metabolites, and that variable times in storage of samples between incubation and HPLC analysis may also have an effect on the relative quantities of these two substances.

The hydroxylation of the methylcarbamoyl substituent of CBzM and CMM did not appear to affect markedly the stability of the triazene moiety. Thus, the metabolites of CBzM were stable for over 1 hr of incubation when microsomal enzymatic activity was blocked by cytochrome P450 inhibitors (Fig. 7). CMM-met1 was stable for up to 10 hr of incubation after the metabolic reaction ceased. Both metabolites, as noted above, were easily isolated in protic solvents. Therefore, one cannot conclude that this hydroxylation step effectively leads to the breakdown of the triazene to form an alkylating agent, and the biological effects of CMM remain unexplained. It is clear, however, that at least in the case of CBzM, further oxidative metabolism occurs. This probably does ultimately lead to the destruction of the triazene, because no other compounds having the appropriate chromophore have been detected. In addition, we do not know that hydroxylation of the methylcarbamoyl substituent is the only route of metabolism of the parent compound. Other possible sites of attack on both the parent compound, and the metabolites, most likely include the chloroethyl group and the benzyl (CBzM) or triazene methyl (CMM) groups. Hydroxylation at these sites would likely lead to dealkylation and destabilization of the triazene moiety. Studies are presently underway to

search for the likely products of such reactions. It is notable, however, that in neither case would an alkyldiazonium ion be the expected result. Consistent with this view are the results of preliminary studies that fail to demonstrate significant levels of chloroethylation or methylation of DNA in the livers of rats treated with single doses of either CBzM or CMM, when CMC clearly caused measurable DNA alkylation under the same conditions.

The final mechanism of (methylcarbamoyl)triazene decomposition remains unclear, but it appears that oxidation and not deacylation is the major metabolic route. Although capable of generating an alkyldiazonium ion upon prolonged incubation under physiological conditions *in vitro*, the methylcarbamoyltriazenes likely act via this totally different, and initially unexpected mechanism *in vivo*. The elucidation of this mechanism may provide valuable insight for the design of new antitumor agents with reduced toxicity.

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