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S-VINYL HOMOCYSTEINE, AN ANALOG OF ETHIONINE THAT IS HIGHLY MUTAGENIC FOR S. TYPHIMURIUM TA100

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SUMMARY

S-Vinyl homocysteine was synthesized as a possible proximate carcinogenic metabolite of ethionine. Unlike ethionine, S-vinyl homocysteine and its N-acetyl methyl ester were directly mutagenic for Salmonella typhimurium TA100; they induced 15-20 and 0.2 revertants/nanomole, respectively. Microsome-cytosol preparations from rat liver increased the mutagenicity of the amide-ester derivative but not that of S-vinyl homocysteine. Methionine inhibited the mutagenic action of S-vinyl homocysteine; other inhibitors of ethionine carcinogenesis did not. Neither ethionine analog was mutagenic for S. typhimuirium TA98.

Ethionine, a naturally occurring (1) S-ethyl analog of methionine, is a methionine anti-metabolite and hepatocarcinogen in the rat (2,3). On the basis of incorporation of ^{14}C and ^{3}H from the S-ethyl group into liver protein, DNA, and RNA (3-6) and the isolation of degradation products of DNA and tRNA that co-chromatographed with ethylated bases (6,7), it has been presumed that ethionine is converted *in vivo* to an ethylating agent that is an ultimate carcinogen. S-Adenosylethionine, a major metabolite of ethionine in the rat (3,5,8), was implicated in early studies as a critical intermediate for reaction with DNA and RNA and for the toxicity and carcinogenicity of ethionine (3). However, some subsequent studies suggested that S-adenosyl-ethionine might not be an ultimate carcinogenic metabolite of ethionine (7,9). Furthermore, administration of cupric acetate inhibits the carcinogenicity of ethionine (10) and the incorporation of radioactivity from S-ethyl-labelled ethionine into macromolecules (11,12), but it causes an increase in the hepatic S-adenosylethionine concentration (13). Methionine and 1,10-

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phenanthroline, two other inhibitors of ethionine toxicity and carcinogenesis, cause a small decrease or no consistent change, respectively, in the levels of hepatic S-adenosylethionine (14-17).

The recent finding of the much higher carcinogenic activity of vinyl carbamate as compared to that of ethyl carbamate (18) encouraged us to synthesize S-vinyl homocysteine (vinthionine) as a possible proximate carcinogenic metabolite of ethionine.

MATERIALS AND METHODS

Vinthionine, a new compound, was prepared by reaction of acetylene with D,L-homocysteine sodium thiolate by a modification of the general method of Prilezhaeva et.al. (19). D,L-Homocysteine (Sigma) (20 g) was dissolved in 400 ml liquid ammonia. Sodium was added in small portions until the blue color persisted for 10 min; the color was dispelled by the addition of a few crystals of ammonium iodide. The ammonia was evaporated under argon and the homocysteine sodium thiolate was dissolved in 300 ml of dry dimethyl sulfoxide. Acetylene was bubbled through the solution for 35 min, and the resulting syrup was neutralized, while being cooled, with 0.5 N HCl. Vinthionine crystallized at 4°. The crystals were washed with cold water, methanol, acetone, and ether and dried *in vacuo*. Yields averaged 60% based on homocysteine.

For the synthesis of N-acetyl vinthionine methyl ester, 4 g of N-acetyl homocysteine methyl ester, prepared from N-acetyl D,L-homocysteine thiolactone (Sigma) (20), in 5 ml of dimethylformamide was added dropwise to 40 ml of acetylene-saturated dimethylformamide containing 0.27 g KOH. Acetylene was bubbled through the solution for 18 hr at room temperature. After dilution with 1 vol. of water, the reaction mixture was extracted with 2 vol. of chloroform. The chloroform phase was reduced to a thick oil under reduced pressure. White crystals, obtained by shaking the residue with 3 ml of water, were washed with Skellysolve B and dried *in vacuo*. Recrystallization from acetone:Skellysolve B (1:4) gave pure N-acetyl vinthionine methyl ester in 9% yield based on N-acetyl homocysteine thiolactone.

For both compounds the elemental analyses (Huffman Laboratories, Wheatridge, Co.) for C, H, N, O, and S were within 0.4%. The infrared spectra (Beckman IR-10) showed the following absorption bands: Vinthionine (KBr) 3100-2500s (broad), 1660s, 1650s, 1640s, 1620s, 1580s, 1511m, 1442m, 1408s, 1337s, 950m, 860m, 537m, 425m. N-Acetyl vinthionine methyl ester (KBr) 3240s, 3050m, 2950m, 1740s, 1633s, 1580m, 1542s, 1431s, 1369s, 1300s, 1203s, 1155s, 939m, 855m, 700s, 585m, 505m, 410m. NMR analysis (Brüker 90 MHz) gave the following peaks (δ,TMS): Vinthionine (DMSO) 1.93, multiplet; 2.81, triplet; 3.31, triplet; 5.15, AB portion of an ABX pattern; 6.48, X portion of an ABX pattern. N-Acetyl vinthionine methyl ester ($CDCl_3$) 2.04, singlet; 2.10, multiplet; 2.72, multiplet; 3.77, singlet; 4.70, multiplet; 5.19, AB portion of an ABX pattern; 6.31, X portion of an ABX pattern. Analysis of the mass spectra (Varian CH-7) showed the expected molecular ions. Vinthionine and its amide ester have absorption maxima at 225 nm with extinctions of 5600 and 5450, respectively.

Mutagenicity assays were carried out with Salmonella typhimurium TA100 and TA98 (21,22). The activating system contained 2 mg of liver microsome-cytosol (S-13) protein from Aroclor 1254-treated Fischer rats. To determine the inhibition of vinthionine mutagenicity by D,L-methionine or D,L-ethionine, one of the latter compounds and vinthionine (60 nmoles/plate) were both added to the molten agar immediately before it was poured on the plate.

The carcinogenic potential of vinthionine was tested in a preliminary experiment in female A/JAX mice (Jackson Laboratory, Bar Harbor, Me.). Groups of 16 to 17, 8 week-old mice were housed, 4 mice per cage, in wire-bottomed cages and fed Wayne Breeder Blox (Allied Mills, Chicago, Ill) and tap water ad libitum. Two i.p. injections of 0.35 mg of vinthionine or ethionine/g body weight/0.03 ml 0.85% NaCl were given in the 1st week. Thereafter, two groups received 0.17 mg of vinthionine or ethionine/g body weight twice weekly for 6 wk (total dose, 2.8 mg/g body weight), and 2 groups received 0.35 mg of the amino acid/g body weight once weekly for 11 wk (total dose, 4.55 mg/g body weight). A positive control group received a single i.p. injection of ethyl carbamate (0.5 mg/g body weight/0.03 ml solution). The mice were killed at 8 months for enumeration of lung adenomas.

RESULTS AND DISCUSSION

Vinthionine induced 15-20 revertants of Salmonella typhimurium TA100 per nanomole with or without the addition of a rat liver metabolizing system (Fig. 1). This striking mutagenic activity of vinthionine is in sharp contrast to the lack of demonstrable mutagenicity for ethionine in this system, either in the presence or absence of Aroclor 1254-induced rat liver microsomes and cytosol (23). However, ethionine is reported to be mutagenic for Coprinus lagopus and Ustilago maydis (24,25).

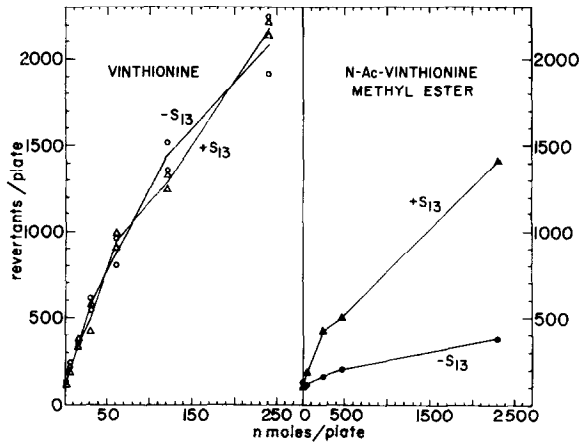

Fig. 1. The mutagenicity of D,L-vinthionine and N-acetyl vinthionine methyl ester for S. typhimurium TA100 in presence or absence of hepatic S-13 fraction from Aroclor 1254-treated rats. Each point represents the datum for a single plate; where data are given for replicate plates, the line is drawn through the average value.

The N-acetyl methyl ester of vinthionine is also mutagenic for strain TA100 without a liver activating system, but it is a much weaker mutagen (220 revertants/μmole, Fig. 1). Addition of rat liver S-13 enhanced the mutagenicity of the amide ester; at high levels of liver S-13 the mutagenicity of the amide ester approached that of vinthionine. Enzymatic hydrolysis of the amide ester to vinthionine is probably the basis of this observation. Neither vinthionine nor its amide ester showed mutagenic activity for S. typhimurium TA98, a frame-shift detecting strain,either with or without the rat liver S-13.

The hepatic toxicity and carcinogenicity of ethionine for the rat are strongly inhibited by addition of methionine to the diet (3,15). Similarly, a several-fold excess of methionine inhibited the mutagenic activity of vinthionine (Fig. 2). Addition of 230 nmoles/plate of DL-methionine reduced the number of revertants induced by 60 nmoles/plate of vinthionine by 50%. At 1000 nmoles/plate methionine completely inhibited the mutagenicity of this level of vinthionine. No toxicity to the bacteria was evident for these

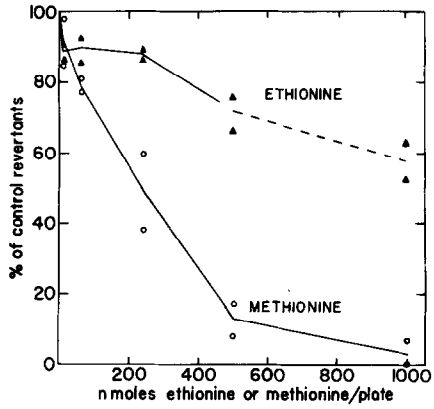

Fig. 2. The effects of the simultaneous addition of D,L-methionine or D,L-ethionine to the top agar on the mutagenicity of D,L-vinthionine (60 nmoles/plate). The response to the addition of 500 or 1000 nmoles of ethionine is denoted by a dotted line, since these high levels of ethionine were toxic. The % of control revertants for each point was calculated as the (net revertants for the treated plates/net revertants for plates without methionine or ethionine) X 100. Each point represents the datum for a single plate, and the lines are drawn through the average values.

doses of methionine, vinthionine, or the combination of the two amino acids. These data suggest that the bacteria may convert the vinthionine to a mutagen through a metabolic pathway that utilizes methionine. Although ethionine is apparently metabolized by the same enzymes that utilize methionine (3,5), ethionine did not inhibit the mutagenicity of vinthionine (Fig. 2). However, the possible inhibition by ethionine of vinthionine mutagenicity could not be studied over a wide range. As evidenced by the reduced numbers of spontaneous revertants and disruption of the bacterial lawn, ethionine was toxic at levels in excess of 500 nmoles/plate. Methionine was not mutagenic under the conditions of these experiments.

Other compounds that modulate the carcinogenicity of ethionine or the metabolism of methionine did not inhibit the mutagenicity of vinthionine for strain TA100. Thus, adenine, cupric acetate, and 1,10-phenanthroline, all of which inhibit the toxicity or carcinogenicity of ethionine (3,10,14,16), were inactive at levels of up to 1000 nmoles/plate as inhibitors of vinthionine mutagenicity. Two inhibitors in rat liver and microorganisms of the formation of S-adenosylmethionine, O-acetylserine and 1-aminocyclopentanecarboxylic acid (26), also had no protective effect. 3-Methylthiopropionic acid, reported to inhibit the major pathway for oxidative metabolism of methionine and ethionine to CO_2 in the rat (27), similarly did not inhibit the mutagenicity of vinthionine.

The unique activity of the S-vinyl derivative, vinthionine, was indicated by the lack of mutagenicity for strain TA100 of several non-amino acid analogs. Ethyl vinyl sulfide, ethyl vinyl sulfone and diethyl sulfone were not mutagenic, with or without the addition of rat liver S-13, at levels up to 2250 nmoles/plate. No mutagenicity was detected for divinyl sulfone, but its toxicity restricted assays to levels below 0.4 nmoles/plate. Other approaches to the mechanisms of mutagenesis by vinthionine are being examined.

Since the induction of lung adenomas in strain A mice has been used as a sensitive and rapid assay for carcinogenicity of some classes of chemicals,

Table 1

Assay of D,L-Vinthionine and D,L-Ethionine for the Induction of Lung Adenomas in A/JAX Mice

See Materials and Methods for protocol.

Compound	Total dose (mg/g body weight)	No. of mice killed at 8 months	No. of mice with lung adenomas	Av. lung adenomas per mouse
Vinthionine	2.80	14	6	0.4
Vinthionine	4.55	15	3	0.3
Ethionine	2.80	11*	4	0.4
Ethionine	4.55	10*	1	0.1
Ethyl carbamate	0.50	16	16**	9.5**
(Solvent only)		16	3	0.2

* The survivals in the mice treated with ethionine were compromised by death of 7 of 17 mice given the higher level during the treatment period and by the loss of 5 mice given the lower dose from acute respiratory infection shortly before the end of the experiment. Only the lungs of the mice killed at 8 mo could be evaluated for lung adenomas.

** Statistically significant ($p < 0.05$) by a χ^2 test. Other values were not significantly different from the solvent control.

vinthionine and ethionine were examined in this test. Neither compound caused significant increases in the percentage of A/JAX mice that developed lung adenomas nor in the average number of lung adenomas per mouse (Table 1). However, the carcinogenicity of ethionine has so far been demonstrated only in liver, and other analyses of the possible relationship of this highly mutagenic analog of ethionine to ethionine carcinogenesis and metabolism are currently in progress.

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