Mutagenicity of the Naturally Occurring Carcinogen Cycasin and Synthetic Methylazoxymethanol Conjugates in Salmonella typhimurium

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ABSTRACT

The aglycone methylazoxymethanol of the naturally occurring carcinogenic glucoside, cycasin, has previously been shown to be mutagenic, but cycasin per se has not. In this work, cycasin was demonstrated to be mutagenic using a modification of the Ames Salmonella test in which it was preincubated with β-glucosidase and the tester strain in liquid medium. The mutagenicity of cycasin to six histidine-dependent Salmonella strains varied considerably with strain HisG46 being the most susceptible. Methylazoxymethyl-β-D-glucosiduronic acid, which also is nonmutagenic per se, similarly became mutagenic when preincubated with β-glucuronidase. Methylazoxymethyl acetate, which is slightly mutagenic by the Ames standard pour plate method, became highly mutagenic on preincubation. The mutagenicity of free methylazoxymethanol was confirmed, and a linear dose-response relationship was observed. The common conditions required for activation of nonmutagenic methylazoxymethanol conjugates, the glucoside cycasin and methylazoxymethyl-β-D-glucosiduronic acid, are 90-min preincubation at 30°, pH 6.5, with an appropriate hydrolase and Salmonella typhimurium HisG46.

INTRODUCTION

Cycasin, the β-D-glucoside of MAM, is a toxic constituent of Cycas circinalis and Cycas revoluta (14) and has been shown to induce tumors in liver, kidney, and large intestine when administered p.o. to conventional animals (4), but it is not carcinogenic in germ-free animals (6). The β-glucosidase produced by the intestinal microflora hydrolyzes the glucoside to release the aglycone MAM. The proximate carcinogen has been identified as the aglycone MAM (9), and repeated i.p. injection of MAM produced tumors in liver, kidney, duodenum, colon, and rectum (5, 6). The aglycone has also been demonstrated qualitatively to be mutagenic to Salmonella (15). A synthetic source of MAM is available as the acetate ester (8) which is also carcinogenic (6) but has only slight mutagenic activity.

It has been postulated that, in the liver, parenterally injected MAM forms the glucosiduronic acid, MAM-GlcUA, which is secreted in the bile and that, in the intestinal tract, it is then hydrolyzed by bacterial β-glucuronidase to free MAM that induces tumors in the duodenum, colon, and rectum (17). The MAM-GlcUA has been synthesized, but its biological effects have not been studied.

Recently, an in vitro bacterial mutagenicity assay has been developed that can be used for rapid evaluation of mutagens and carcinogens (16). In this assay, a rat liver microsomal enzyme mixture designated S-9 mix is used for conversion of some compounds to their active form. Although the aglycone MAM itself has been shown to be mutagenic to Salmonella typhimurium, the glucoside, cycasin, was not mutagenic with this test system (10, 15).

This paper reports that, on preincubation of cycasin with β-glucosidase and of MAM-GlcUA with β-glucuronidase, these compounds are converted to mutagenic forms. The action of the hydrolases simulates the role of enzymes produced by the microflora of animals given cycasin p.o. Increase in the mutagenicity of MAM-OAc by preincubation of the compound with the test organism is also reported.

MATERIALS AND METHODS

Bacterial Tester Strains. Strains HisG46, TA92, TA1975, TA1950, TA1535, and TA100 of S. typhimurium were used. These tester strains are histidine auxotrophs which detect base-pair change mutation. S. typhimurium HisG46, TA92, and TA1975 have the capacity for excision repair of DNA damage produced by mutagens, but TA1950, TA1535, and TA100 have no capacity for excision repair (1). TA92 and TA100 were derived from HisG46 and TA1535, respectively, by introduction of the R-factor plasmid pKM101 (11).

Chemicals. Cycasin was isolated from C. circinalis seeds by the method of Nishida et al. (14). MAM was prepared by the method of Kobayashi and Matsumoto (3). MAM-GlcUA was synthesized by one of the authors (H. M.) by catalytic oxidation of cycasin. MAM-OAc was obtained from Ash-Stevens, Inc., Detroit, Mich. β-Glucosidase (almond, 4.7 units/mg) and β-glucuronidase (Escherichia coli, 90 units/mg) were purchased from Sigma Chemical Co., St. Louis, Mo.

Mutagenicity Test. The standard pour plate method of Ames et al. (1) and the preincubation method of Yahagi et al. (18) were used. For cycasin and MAM-GlcUA, the procedure was modified by including a longer period of preincubation of the test organism with the test compound and an appropriate hydrolase.

Cycasin was preincubated at 30° with β-glucosidase (30 units, 6.3 mg protein) in 100 mM phosphate buffer, pH 6.5,
with 1 to 2 x 10^8 cells of the bacterial tester strain in a total volume of 0.65 ml with mechanical shaking. Various incubation periods were used. After incubation, 2 ml of molten top agar (45°C) containing 50 μM L-histidine and biotin were added, and the mixture was poured over a plate of 30 ml of minimal-glucose agar in a Petri dish. After 2 days at 37°C, the number of histidine-independent revertant colonies was scored. The total number of revertants above 2000 was determined by counting the number in 10 areas under a dissecting microscope and multiplying the average number by the ratio of the area examined under the microscope to the total area of the plate. For determination of the toxicity of the test compound, the growth of the background lawn on each plate was examined with a dissection microscope.

MAM-GlcUA (5 to 40 μmol) was preincubated with β-glucuronidase (2000 units, 4 mg protein) and the tester strain in the same way as in tests on cycasin. MAM-OAc was preincubated with the test organism alone at 30°C in 100 mM phosphate buffer at pH 7.4 for 30, 60, and 90 min. MAM was tested by the preincubation method with and without S-9 mix.

S-9 mix was prepared by a slight modification (12) of the method of Ames et al. (1).

RESULTS AND DISCUSSION

Cycasin. Cycasin was not mutagenic by the Ames standard pour plate method (1) even with S-9 mix, but it became mutagenic when preincubated with almond β-glucosidase and the tester strain in liquid suspension.

The optimum pH of the β-glucosidase determined with p-nitrophenyl-β-D-glucoside as substrate was 5.5, but the survival of Salmonella rapidly decreased below pH 5.5. Thus, preincubation was carried out at pH 6.5 to avoid decrease in the survival of the Salmonella. The number of revertants increased linearly with increase in the amount of β-glucosidase added to the preincubation mixture reaching a maximum with about 30 units of β-glucosidase per preincubation mixture containing 10 μmol of cycasin. The number of revertants varied with the length of the preincubation time, and a preincubation period of 90 min was found to result in a large number of revertants (Chart 1).

The mutagenic response of Salmonella to cycasin preincubated with β-glucosidase varied considerably with the strain of histidine mutant used. Six strains (HisG46, TA92, TA1975, TA1950, TA1535, and TA100) were tested. HisG46, which has the capacity for excision repair, gave the best response; TA1950, TA1535, and TA100, which have no capacity for excision repair, gave weak responses; and TA92, which gave a moderate response with up to 15 μmol of cycasin, was killed by higher concentrations (Chart 2).

MAM-GlcUA. Like cycasin, MAM-GlcUA was not mutagenic to Salmonella but, when preincubated with E. coli β-glucuronidase, it became mutagenic to HisG46 as shown in Chart 3. The number of revertants was much greater on incubation for 90 min than on incubation for 30 or 60 min.

MAM-OAc. By the Ames standard pour plate method with HisG46, 30 μmol of MAM-OAc induced 150 revertants without S-9 mix; but when preincubated with the bacterial cells for 90 min, the same amount of MAM-OAc induced 11,000 revertants. There was a linear increase in revertants with increase in the quantity of MAM-OAc from 10 to 50 μmol/plate. The mutagenic effect also increased with increase in the incubation time as shown in Chart 4.

MAM. As expected, MAM was mutagenic to Salmonella. It is a direct-acting mutagen and requires no activation. It gave a linear dose-mutagenicity response curve at doses of 10 to 20 μmol, but it was cytotoxic in quantities greater than 25 μmol. In the presence of S-9 mix, it had no killing effect, and its mutagenicity increased 5- to 8-fold. MAM readily methylates a number of compounds including proteins (7, 13). Extensive methylation of bacterial DNA and proteins results in death of the organism, but the presence of S-9 mix apparently reduces this cytotoxicity. Of the strains tested, strain HisG46 was the

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noncarcinogenic (2, 4). Cycasin becomes mutagenic only when preincubated with β-glucosidase. This finding supports the idea that the carcinogenicity of p.o. cycasin in animals is due to its hydrolysis by β-glucosidase produced by the intestinal microflora. There is no report on the carcinogenicity of MAM-GlcUA but, since this compound is hydrolyzed to MAM by E. coli β-glucuronidase, it can be predicted that it will be found to be carcinogenic when administered p.o. to animals.

Preincubation of tester strain, test compound, S-9 mix or buffer, and appropriate glycosidases is recommended for the mutagenicity testing of glycosides suspected of being carcinogenic.

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REFERENCES


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