ABSTRACT

We examined the metabolism of 14C-labeled methylazoxymethanol (MAM) in male F344 rats pair-fed for 21.0 days either a liquid control diet, an isocaloric liquid diet containing 6.6% ethanol by volume (continuous ethanol diet), or the ethanol diet for 20.5 days followed by the control diet for 0.5 day (interrupted ethanol diet). Compared to rats fed the control liquid diet, metabolism of [1,2-14C]MAM acetate to exhaled 14CO2 was inhibited by 25 to 42% in rats fed the continuous ethanol diet, but was initially stimulated by 90% in rats given the interrupted ethanol diet. MAM-induced DNA methylation, as reflected in 7-methylguanine and O6-methylguanine content 24 h after carcinogen administration, was inhibited in the colon mucosa of rats fed the interrupted ethanol diet by 52 to 54%, and an even greater inhibition (71 to 86%) of DNA methylation occurred in the colon mucosa of rats fed the continuous ethanol diet. Liver DNA methylation was significantly inhibited (by 32 to 42%) only in those rats fed the continuous ethanol diet. Liver microsomes isolated from rats fed the 3 diets metabolized MAM to formaldehyde and methanol in vitro, but liver microsomes from rats fed the continuous ethanol diet were 12 to 15 times more active than liver microsomes from rats fed the control diet. Liver microsomes isolated from rats fed the interrupted ethanol diet were only 3 to 5 times more active in MAM metabolism than liver microsomes from rats fed the control diet, indicating very rapid turnover of the ethanol-induced enzyme(s) catalyzing the oxidation of MAM. Although chronic ethanol feeding enhanced the activity of liver microsomes for MAM metabolism, ethanol was found to inhibit the reaction competitively. Hepatocytes isolated from rats fed the continuous ethanol diet were considerably more sensitive to MAM-induced unscheduled DNA synthesis than hepatocytes isolated from rats given the control liquid diet, indicating that the stimulation of MAM metabolism by dietary ethanol results in increased DNA damage, observable in an in vitro system. Thus, the increased metabolic activation of MAM, due to enzyme induction by ethanol which is observed in vitro, is not reflected in increased liver DNA methylation in vivo. In addition, the great degree of similarity between the present observations involving MAM and the results of our previous studies involving azoxymethane and N-nitrosodimethylamine (O. S. Sohn et al., Cancer Res., 47: 3123-3129, 1987) raises the question of whether all three carcinogens might not be metabolically activated by the same form of ethanol-induced rat liver microsomal enzyme(s).

INTRODUCTION

MAM, a potent colon and liver carcinogen (1-3), is metabolically derived from the widely used model colon carcinogen, 1,2-dimethylhydrazine, by way of the intermediates, azomethane and azoxymethane (4-6). MAM also occurs in nature as the aglycone moiety of the liver and intestinal carcinogen, cycasin (1), as well as of the related compound, macrozamin (7). Nonconjugated MAM is an unstable compound (8) with a half-life of approximately 12 h under physiological conditions (9). Although its spontaneous decomposition yields a reactive species, presumably the methyldiazonium ion (4, 8), which is capable of alkylating nucleic acids (10), there is adequate evidence that the in vivo production of a DNA-methylating species from MAM is enzymatically catalyzed (11-13). The metabolic formation of MAM from 1,2-dimethylhydrazine, and the postulated routes whereby the methyldiazonium ion is formed by the spontaneous breakdown (4, 8) of MAM as well as by enzymatically catalyzed (9, 12) oxidation, are illustrated in Fig. 1. The oxidation of MAM has been identified as the rate-limiting step in the metabolic activation of 1,2-dimethylhydrazine and azoxymethane (14); thus, characterization of enzymes catalyzing the activation of MAM and of factors modifying the activity of these enzymes is important not only towards understanding the mechanisms of action of these three closely related carcinogens, but also towards their more rational and more efficient application in laboratory experiments intended to model the human condition.

In a previous study (15), we found that the chronic administration of a liquid diet containing 6.6% ethanol by volume to F344 rats led to a 12- to 18-fold induction of liver microsomal enzymes catalyzing the hydroxylation of azoxymethane to MAM, as well as to an equal induction of N-nitrosodimethylamine demethylase. Since liver microsomes from deer mice (12) as well as from F344 rats (13) have previously been shown to actively metabolize MAM, we sought to determine, in the present work, whether chronic ethanol feeding would similarly result in increased ability of rat liver microsomes to carry out the in vitro metabolism of MAM and, further, to determine to what extent such effects would be reflected in the in vivo metabolism of the carcinogen and its ability to methylate DNA in the liver and colon mucosa.

MATERIALS AND METHODS

Chemicals. MAMOAc was purchased from Starks Associates, Buffalo, NY. 14C-labeled methanol, formaldehyde, and formic acid, for use as chromatographic standards, and [1,2-14C]MAMOAc were obtained from NEN Research Products, Boston, MA. The latter compound was purified to >99% radiochemical purity by chromatography on silica gel (13). [1,2-14C]MAM was prepared by hydrolyzing [1,2-14C]MAMOAc with porcine liver esterase (Sigma Chemical Co., St. Louis, MO); otherwise the conditions of hydrolysis were essentially the same as described by Feinberg and Zedek (9). Prior to use as substrate in microsomal incubation mixtures, labeled MAM was freed of decomposition products ([14C]methanol and [14C]formaldehyde) by HPLC on a Whatman Magnum 9 ODS column eluted with water. The product was stored for periods of no longer than 2 wk at -20°C prior to its use as substrate.

Animals and Diets. Male F344 rats, 10 wk old, average body weight approximately 180 g, were obtained from Charles River, Inc., Wilmington, MA. The animals were divided into 3 groups. As described in detail previously (15, 16), one group ("continuous ethanol diet
group") received a 6.6% ethanol liquid diet containing ethanol as 33% of the calories (Bioserv rat Liquid Diet 16; Bioserv, Inc., Frenchtown, NJ) for exactly 21.0 days; a second group ("interrupted ethanol diet group") was fed the same diet for 20.5 days and then transferred to an equicaloric ethanol-free control diet (Bioserv rat Liquid Diet 15) for 0.5 days (from 9:00 p.m. to 9:00 a.m.), and a third group ("control diet group") was pair fed the ethanol-free equicaloric liquid diet for 21.0 days. In all cases, the liquid diets served as the sole sources of calories and nutrients. After 21.0 days on their respective diets, the animals were used either for in vivo metabolism studies of labeled MAMOAc, or were sacrificed to obtain liver microsomes for in vitro experiments. At this time, rats in the continuous ethanol diet and interrupted ethanol diet groups weighed an average of 203 g; those in the control diet group weighed an average of 205 g.

In Vivo Metabolism. Exactly 21 days after commencement of diet administration, rats from the three groups were each given injections of 25 mg/kg of [1,2-14C]MAMOAc, 20 µCi per animal, s.c., and immediately placed into Delmar-Roth-type glass metabolism cages (Bioserv, Inc., Frenchtown, NJ). Air, dried and freed of CO2, was drawn through the cages at a rate of approximately 250 ml/min by means of peristaltic pumps. The air exiting each cage was passed through 2 serially connected gas washers containing 1 M NaOH, which washers served to retard the elution of formic acid, a metabolite of MAM (13), which would otherwise coelute with methanol.

Determination of Methylated Guanines in Liver and Colon Mucosa DNA. DNA was isolated by the method of Margison and Kleihues (17), hydrolyzed for 18 h in 0.1 M HC1 at 37°C, and analyzed for O'-MeG by the HPLC-fluorescence detection method of Herrón (18). Results are expressed as the mean ± SD of grain counts for 3 coverslips. In this assay, compounds yielding net mean nuclear grain counts above 5 grains per nucleus, which value represents the upper limit of control, are considered significantly positive in inducing UDS.

RESULTS

In Vivo Metabolism of [1,2-14C]MAMOAc. The time courses for the overall metabolism of [1,2-14C]MAMOAc, 25 mg/kg, s.c., to exhaled 14CO2 by rats fed either the ethanol diet for 21.0 days, or the ethanol diet for 20.5 days and the control diet for 0.5 day, or the control liquid diet for 21.0 days, are shown in Fig. 2. With respect to rats in the control diet group, rats in the continuous ethanol diet group were found to exhale 58 to 75% less 14CO2 (P < 0.001) in the first 7 h after [1,2-14C]-MAMOAc administration, indicating pronounced inhibition of MAM metabolism during this period. Even after 24 h, the total amount of 14CO2 exhaled by the ethanol-fed rats was still approximately 24% less than that exhaled by the rats fed the control diet.

In contrast, metabolism of [1,2-14C]MAMOAc by rats in the interrupted ethanol diet group was more rapid in the first hour

- 6-phosphate dehydrogenase, 1.5 mM NADP+, 3.5 mM MgCl2, 0.1 mM potassium phosphate buffer, pH 7.0, labeled MAM, and approximately 1 mg microsomal protein in a total volume of 0.5 ml.

After incubation at 37°C in a shaker bath, the reaction vessels were quickly chilled to 0°C. The suspensions were clarified and deproteinized by centrifugal ultrafiltration (600 x g, 30 to 60 min, 0–4°C) using Centricon Micropartition system tubes (Amicon, Danvers, MA). Aliquots of the ultrafiltrates were submitted to HPLC using 2 Whatman ODS-3 (0.46 x 25 cm) columns in series, preceded by a 0.7- x 5-cm column packed with Aminex A-29 anion-exchange resin (Bio-Rad Labs., Rockville Centre, NY) in the phosphate form. The column system was eluted with 0.2 M ammonium phosphate buffer, pH 3.3, at a flow rate of 0.5 ml/min for the first 28 min, and at 1 ml/min thereafter. The absorbance of the effluent was monitored at 215 nm, and fractions were collected at 1-min intervals for determination of radioactivity by scintillation counting. The anion-exchange precolunm served to retard the elution of formic acid, a metabolite of MAM (13), which would otherwise coelute with methanol.

UDS in isolated Hepatocytes. Hepatocytes, isolated from rats fed for 21.0 days the control or ethanol-containing liquid diets, were attached to cover slips as described in detail elsewhere (20, 21), and exposed to [methyl-3H]thymidine (10 µCi/ml, 49 Ci/mmol) and various concentrations of MAM for 20 h at 37°C. After incubation, the cultures were washed, the coverslips were mounted on glass slides, dipped in photographic emulsion, and exposed for 14 days at 4°C. Results are expressed as the mean ± SD of grain counts for 3 coverslips. In this assay, compounds yielding net mean nuclear grain counts above 5 grains per nucleus, which value represents the upper limit of control, are considered significantly positive in inducing UDS.

![Fig. 2. Metabolism of [1,2-14C]MAMOAc to exhaled 14CO2 by F344 rats fed either the continuous ethanol diet for 21 days (“ethanol diet”), the control isocaloric liquid diet for 21 days (“control diet”), or the ethanol diet for 20.5 days followed by the control liquid diet (“interrupted ethanol diet”). All rats were given injections of 25 mg/kg[1,2-14C]MAMOAc, s.c., exactly 21.0 days after the feeding regimen was begun. During the metabolim study, the respective diets were available to the rats ad libitum. Bars; SE; asterisks, significant differences (P < 0.05 or less) from rats fed the control diet continuously.](image)
or two after carcinogen administration compared to rats fed the control diet. Thus, in the first hour following [1,2-14C]MA-MOAc treatment, rats on the interrupted ethanol diet schedule exhaled approximately 90% more 14CO2 (P < 0.05) than did the rats maintained on the control diet. Although the effect appeared to persist for several hours, the differences between the two groups ceased to be statistically significant after the first hour. During the 24 h in the metabolism cages, rats in the ethanol diet group each consumed 9.5 ± 4.2 ml of diet, the rats in the interrupted ethanol diet group each consumed 13.5 ± 8.3 ml, and the rats in the control diet group each consumed 52.5 ± 2.9 ml.

Metabolism of [1,2-14C]MAM by Liver Microsomes. The metabolite patterns which resulted from the incubation of [1,2-14C]MAM, cofactors, and liver microsomes either from rats in the control diet group or from rats fed the ethanol diet continuously for the same period, are shown in Fig. 3. As previously reported for uninduced rat liver microsomes (13), liver microsomes from rats fed the continuous ethanol diet as well as liver microsomes from rats fed the interrupted ethanol diet (results not shown) metabolized MAM to methanol and formic acid. This is in contrast to the spontaneous decomposition of the carcinogen, which yields methanol and formaldehyde (18). It is clear that liver microsomes obtained from rats fed the ethanol diet were significantly more active in metabolizing MAM than were microsomes obtained from livers of rats fed the control diet. In the HPLC profiles shown in Fig. 3, the analytical conditions were altered slightly from those previously used (13, 15), in that the pH of the HPLC eluant was increased from 3.1 to 3.3. This small increase in pH further retarded the elution of labeled formic acid (pK_a = 3.77), enabling us to detect a new minor peak, eluting at approximately 20 ml, upon analysis of incubation mixtures containing liver microsomes from ethanol-consuming rats. Preliminary experiments suggest that this peak may be due to [14C]methylphosphate, the reaction product of the alkylating species derived from labeled MAM with phosphate buffer in the incubation medium.

The enhanced activity of liver microsomes from ethanol-consuming rats for the metabolism of MAM is evident in Fig. 4, which compares the initial rates of this reaction, catalyzed by liver microsomes from rats subjected to the 3 different dietary regimens. With respect to liver microsomes from rats continuously fed the control liquid diet, the rate of MAM metabolism by liver microsomes from rats continuously fed the ethanol diet for 21.0 days was increased 12- to 15-fold (P < 0.001). Liver microsomes obtained from rats submitted to the interrupted ethanol feeding regimen of 20.5 days on the ethanol diet and 0.5 days on ethanol-free liquid diet prior to sacrifice also showed enhanced ability to metabolize MAM; however, the magnitude of this increase with respect to control microsomes was only 3- to 4-fold (P < 0.01). A parallel effect of continuous and interrupted ethanol feeding on the enhancement of azoxymethane and \( N \)-nitrosoethylamine metabolism by rat liver microsomes was observed previously (15).

Inhibition of MAM Metabolism in Vitro by Ethanol. In previous work, we found that the metabolism of azoxymethane by ethanol-induced as well as by noninduced rat liver microsomes was competitively inhibited by ethanol (15). This effect appears to be entirely analogous to the competitive inhibition of microsomal \( N \)-nitrosoethylamine demethylase by ethanol which has been described by others (22). As shown by plots relating the reciprocals of initial reaction rates and MAM concentrations in the presence of differing concentrations of ethanol in Fig. 5, ethanol is also a competitive inhibitor of the metabolism of MAM by liver microsomes from rats fed the continuous ethanol diet. By plotting the slopes of the double reciprocal plots against ethanol (EtOH) concentration. 

![Fig. 3. HPLC profiles of products resulting from incubation of [1,2-14C]MAM and cofactors with liver microsomes from rats continuously fed the control liquid diet (A) and rats continuously fed the ethanol diet (B) for 21.0 days prior to sacrifice. The peak labeled with a question mark probably represents methyl phosphate; however, this has not yet been verified. For conditions, see "Materials and Methods."](image)

![Fig. 4. Time courses of metabolism of [1,2-14C]MAM to labeled formic acid, methanol, and presumed methylphosphate by liver microsomes obtained from rats fed the control liquid diet, the continuous ethanol diet, or the interrupted ethanol diet. The concentration of [1,2-14C]MAM in the incubation mixtures was 1.0 mM. The extent of the reaction was determined by quantitation of products, which were separated by HPLC as in Fig. 3. Conditions of incubations are described in "Materials and Methods." Points, mean of 4 replicate determinations using pooled liver microsomes from 5 or 6 animals; bars, SE.](image)

![Fig. 5. Plots relating the reciprocals of initial rates of metabolism and of MAM concentration. Liver microsomes isolated from rats fed the continuous ethanol diet for 21 days were used. Concentrations of ethanol were: A, 1.0 mM; B, 0.2 mM; and C, 0.0 mM. Inset shows the determination of an approximate value for the apparent \( K_i \) (0.13 mM) of ethanol by plotting the slopes of the double reciprocal plots against ethanol (EtOH) concentration.](image)
[14C]MAM available did not allow replicate determinations to be made; therefore the degree of error in this value is not known.

Effects of Ethanol Feeding on MAM-induced UDS in Isolated Hepatocytes. Table 1 shows the results of 2 separate feeding experiments in which MAM-induced UDS was determined in hepatocytes isolated from rats fed the continuous diet or the control liquid diet for 21 days. Although considerable interexperiment variability is evident, the data nevertheless indicate that hepatocytes isolated from ethanol-fed rats were in each case more sensitive to MAM-induced DNA damage, as evidenced in increased DNA repair processes. This effect is consistent with the enhanced ability of liver microsomes from ethanol-fed rats to metabolize the carcinogen, as described above, and indicates that the increased rate of metabolism of MAM indeed results in the production of greater levels of a DNA-damaging species. Similar stimulations of azoxymethane-induced (15) and N-nitrosodimethylamine-induced (15, 23) UDS in hepatocytes isolated from ethanol-fed rats have been observed previously.

Effects of Ethanol Feeding on MAMOAc-induced DNA Methylation in Vivo. The contents of O-MeG and 7-MeG of liver and colon mucosa DNAs, isolated 24 h after the administration of 25 mg/kg MAMOAc, s.c., to rats fed the ethanol, the interrupted, or the control diets are shown in Fig. 6. Compared to rats fed the control diet, MAMOAc-induced liver DNA methylation was unchanged in rats of the interrupted ethanol diet group, but was inhibited by 32 to 42% (P < 0.003) in rats of the continuous ethanol diet group.

In contrast to MAMOAc-induced DNA methylation in rat livers, DNA methylation was significantly inhibited in the colon mucosae of rats in both the interrupted ethanol diet group, and in the colon mucosae of rats in the continuous ethanol diet group. Thus, in the interrupted ethanol diet group, the extent of colon mucosa DNA methylation was only 46 to 48% (P < 0.03) of the control diet group, and the colon mucosa DNA methylation in the continuous ethanol diet group was 14 to 29% (P < 0.003) of the control diet group. These results indicate that the regimens of continuous and interrupted ethanol feeding have different effects on MAMOAc-induced DNA methylation in rat liver and colon, and that while DNA methylation is inhibited in both organs by continuous ethanol feeding, the effect is much more pronounced in the colon mucosa.

DISCUSSION

The results presented here show that the metabolism of MAM to exhaled CO₂ is significantly inhibited in rats chronically consuming a liquid diet which contains 6.6% ethanol by volume (Fig. 2). This inhibition of metabolism is accompanied by decreased DNA methylation in the liver, and even more so in the colon mucosa (Fig. 6). These results indicate that ethanol is a powerful inhibitor of MAM activation in both the liver and colon mucosa. Even though chronic ethanol intake increases the levels of MAM metabolizing enzymes in the liver endoplasmic reticulum manifold (Figs. 3 and 4), it is evident that, in terms of MAM metabolism in vivo, the inhibitory effects of ethanol outweigh its effects as an enzyme inducer.

 Interruption of the chronic ethanol feeding for 12 h prior to administration of labeled MAMOAc, by giving the rats control liquid diet during this and the subsequent 24-h period, has the effect of partially depleting body ethanol content, even though the levels of the hepatic enzyme(s) of MAM metabolism which had been induced by the ethanol feeding have not yet decreased to basal levels (Fig. 4). Under these conditions, we observed a stimulation of the in vivo metabolism of MAM (Fig. 2), as would be expected. However, no increase was observed in the extent of liver DNA methylation, assayed at 24 h after carcinogen administration, while significant inhibition of DNA methylation occurs in the colon mucosa (Fig. 6). The decreased DNA methylation in the colon mucosa may be due to the increased rate of metabolism of MAM by the liver, causing a lesser availability of MAM for metabolic activation by the colon mucosa. As reflected in the degree of DNA methylation (Fig. 6), about 10 times more MAM is metabolized in the liver than in the colon mucosa of control animals; thus even small variations in the metabolism of MAM by the liver may result in disproportionate changes in MAM metabolism by the colon. In previous work (13) we observed that the selective inhibition of liver metabolism of MAM by 3-methylpyrazole resulted in increased DNA methylation in the colon mucosa, suggesting that as far as the colon is concerned, metabolism of MAM by the liver represents a detoxification process. However, an increase in the rate of metabolism of MAM by the liver would not be expected to result in corresponding increased liver DNA methylation (assayed at 24 h), since essentially all of the MAM administered to rats in the control diet as well as in the interrupted ethanol diet groups appears to be metabolized and/or excreted in the first 6 to 7 h following the administration of the carcinogen (Fig. 2). As pointed out by Hong and Yang (24) in similar studies on induction of N-nitrosodimethylamine dealkylase and N-nitrosodimethylamine metabolism, an increase in the rate of carcinogen metabolism to an alkylating species in vivo does not influence the total extent of DNA alklylation when small, nonsaturating amounts of carcinogen are administered.

While the chronic administration of ethanol to rats resulted in an inhibition of MAM metabolism and of MAM-induced

Table 1 MAM-induced unscheduled DNA synthesis in hepatocytes isolated from F344 rats fed control liquid diet or liquid diet containing 6.6% by volume ethanol for 21 days

<table>
<thead>
<tr>
<th>[MAM]/plate (mm)</th>
<th>Control diet</th>
<th>Ethanol diet</th>
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<tbody>
<tr>
<td></td>
<td>Assay 1</td>
<td>Assay 2</td>
</tr>
<tr>
<td>1.35 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>++ + +</td>
<td>N.D</td>
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<tr>
<td>1.35 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>55.3 ± 13.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.6 ± 43.6</td>
</tr>
<tr>
<td>1.35 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>10.8 ± 1.7</td>
<td>20.8 ± 0.9</td>
</tr>
<tr>
<td>1.35 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>7.9 ± 1.6</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>1.35 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>1.4 ± 2.0</td>
<td>0.2 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two separate assays of MAM-induced UDS were performed at different times. In Assay 1, the hepatocytes used were isolated from 2 rats fed the ethanol liquid diet and from one rat pair-fed the control liquid diet. In Assay 2, hepatocytes were isolated from one rat fed the ethanol diet and one rat pair-fed the control diet.

<sup>b</sup> N.D., not determined.

<sup>c</sup> Mean ± SD of grains/nucleus for 3 coverslips.
liver and colon mucosa DNA methylation in vivo, the treatment also produced a 12- to 15-fold induction of liver microsomal enzymes that metabolize MAM to methanol and formic acid in vitro (Fig. 4). Moreover, hepatocytes isolated from rats chronically fed the ethanol diet exhibited enhanced sensitivity to MAM-induced UDS (Table 1), indicating that the increased rate of MAM metabolism in vitro due to enzyme induction by ethanol is indeed associated with increased DNA damage. In the case of the UDS assay, MAM is present in relatively large, presumably saturating levels that cannot be exhausted by metabolism occurring in the hepatocytes; thus, increased enzymatic activity leads to increased DNA damage, in contrast to the situation in vivo. Thus the enhanced enzymatic activity and DNA damage observed in the in vitro systems are not expressed in parallel effects in vivo.

The effects of continuous and interrupted ethanol feeding on the in vivo and in vitro metabolism of MAM as well as on the stimulation of UDS in isolated hepatocytes that we have presented here strongly resemble in all aspects the effects we have previously observed in closely related experiments on the metabolism of azoxymethane and N-nitrosodimethylamine (15, 16). Because of the striking similarities of the effects of ethanol on the metabolism of these three carcinogens, we suggest, as a working hypothesis, that the same ethanol-induced microsomal enzyme, probably a form of cytochrome P-450 with aniline hydroxylase activity (25), may be active in catalyzing the hydroxylation of azoxymethane, the demethylation of N-nitrosodimethylamine, as well as the oxidation of MAM; all of these reactions resulting in the metabolic activation of the respective carcinogens in vitro, although not necessarily leading to increased DNA methylation in vivo. This proposal is currently being tested.

ACKNOWLEDGMENTS

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Effects of Chronic Dietary Ethanol on *in Vivo* and *in Vitro* Metabolism of Methylazoxymethanol and on Methylazoxymethanol-induced DNA Methylation in Rat Colon and Liver

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