A 7-Hydroxymethyl Sulfate Ester as an Active Metabolite of 7,12-Dimethylbenz[*a*]anthracene

Abstract. *7-Hydroxymethyl-12-methylbenz*[a]*anthracene (7-HMBA), a carcinogenic major metabolite of 7,12-dimethylbenz*[a]*anthracene (DMBA) in liver, was transformed by liver cytosolic sulfotransferase to reactive 7-HMBA sulfate, which is mutagenic toward* Salmonella typhimurium *strain TA98. The mutagenicity of 7-HMBA in the presence of hepatic sulfotransferase was much higher than that of DMBA or 7-HMBA in the presence of hepatic monooxygenase.*

The great increase in carcinogenicity of benz[*a*]anthracene (BA) on the introduction of methyl groups in the 7 and 12 positions ("L region") (*1*) has remained unexplained. 7,12-Dimethylbenz[*a*]anthracene (DMBA) is considered to be a procarcinogen, like many other polycyclic aromatic hydrocarbons, and to exert its carcinogenic activity after being metabolized in the animal body (*2*). Epoxides and diol epoxides have been considered the ultimate reactive forms of the carcinogenic hydrocarbons, including DMBA, since they are all metabolically activated by microsomal monooxygenase and epoxide hydrolase (*1–3*). Recent studies of the covalent bonding of DMBA metabolites to DNA in mouse skin in vivo (*4*) and mouse embryo cells in vitro (*5*), as well as of their carcinogenic (*6*) or mutagenic (*7*) activity, strongly suggested that DMBA-3,4-diol-1,2-epoxide is the most likely ultimate carcinogen. However, it is of interest that DMBA, specifically labeled with ^3H in the 7-methyl group, loses some of the radioactive label on binding to DNA in an in vivo system (*8*). On this basis it was suggested that DMBA is metabolized to 7-hydroxymethyl-12-methylbenz[*a*]anthracene (7-HMBA) before biotransformation to the ultimate form (which was still considered to be related to its diol epoxides) (*9*). 7-HMBA, a potent carcinogen (*10*) with no intrinsic mutagenicity to *Salmonella typhimurium* (*11*), is the major metabolite of DMBA in rat liver (*12*). In this report we provide evidence for the metabolic activation of 7-HMBA to the intrinsic mutagen 7-HMBA sulfate in rat liver cytosol in the presence of a 3′-phosphoadenosine-5′-phosphosulfate (PAPS) generating system. The mutagenicity of 7-HMBA toward *S. typhimurium* strain TA98 was greater in the presence of the hepatic sulfotransferase-PAPS system than that of DMBA or of 7-HMBA in the presence of rat liver microsomes and an NADPH-generating system.

7-HMBA was incubated at *p*H 7.4 for 20 minutes with a dialyzed soluble supernatant fraction of a male Wistar rat liver homogenate in the presence of adenosine triphosphate (ATP), sodium sulfate, magnesium chloride (hepatic sulfotransferase-PAPS system), and a suspension of *S. typhimurium* TA98 or TA100 in 0.1*M* phosphate buffer, *p*H 7.4. Then the mixture was diluted with soft agar and reincubated on hard agar plates, prepared by the method of Ames *et al.* (*13*), at 37°C for 48 hours. His^+ reverse mutations were observed to a significant extent in the TA98 strain (Table 1) but to a very small extent in TA100. The reverse mutation induced by 7-HMBA in TA98 was not observed when only 7-HMBA, the hepatic subcellular fraction, ATP, or sodium sulfate was omitted from the bacterial assay system or when the hepatic fraction was inactivated by heating at 100°C for 10 minutes. The addition of a *Helix pomatia* sulfatase preparation to the mixture decreased the number of His^+ revertant colonies remarkably.

In order to determine whether its sulfate ester was formed as an active metabolite in the bioassay system, 7-HMBA was incubated under the conditions mentioned above in the absence of bacteria. After 20 minutes the mixture was shaken with ether to remove the substrate, and then the aqueous phase was filtered to remove precipitated protein. The filtrate was poured onto an Amberlite XAD-2 column (1 by 5 cm). The column was washed with water and eluted with methanol. The residue obtained on evaporation of the solvent from the methanolic fraction was highly mutagenic toward *S. typhimurium* TA98 in the absence of the hepatic sulfotransferase-PAPS system and weakly active toward the TA100 strain. The direct mutagenicity of the water-soluble metabolite was lost after treatment with sulfatase or 1*N* NaOH. The mutagenic metabolite that was isolated had the same absorption maxima with the same relative absorbances as 7-HMBA in ethanol. It produced a single spot with an intense, bluish violet fluorescence at R_f 0.56 on a thin-layer chromatogram with Merck cellulose F_{254} in *n*-butanol, acetic acid, and water (4:2:1). Under these conditions 7-HMBA produced a fluorescent spot at the solvent front. The metabolite was not formed from 7-HMBA when only one of the components of the sulfotransferase-PAPS system was omitted from the incubation mixture or when the hepatic fraction was heated at 100°C for 10 minutes. Treatment of the metabolite with *H. pomatia* sulfatase or 1*N* NaOH quantitatively formed an ether-extractable material that was identical with 7-HMBA by ultraviolet and mass spectroscopy, thin-layer chromatography, and gas-liquid chromatography.

To obtain further verification that the water-soluble metabolite was 7-HMBA sulfate, the eluate from the XAD-2 column was methylated with an ethereal diazomethane solution (*14*). The methylated product was soluble in *n*-hexane and had a simple mass spectrum with a molecular ion peak at mass-to-charge ratio (*m/e*) 366 and fragment ions at *m/e* 351 ($M^+ - CH_3$), 255 ($M^+ - OSO_3CH_3$),

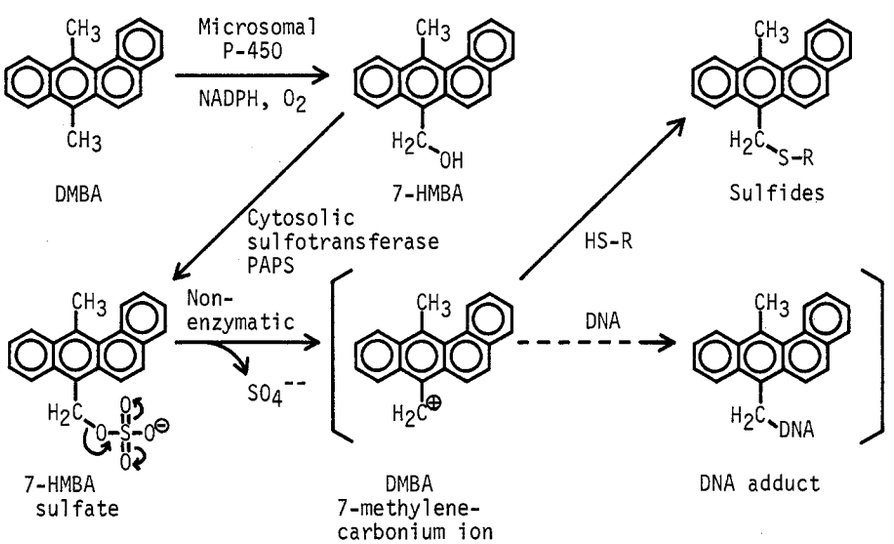

Fig. 1. Hepatic biotransformation of DMBA into the mutagenic 7-HMBA sulfate through 7-HMBA; *HS-R* represents ethyl mercaptan and glutathione.

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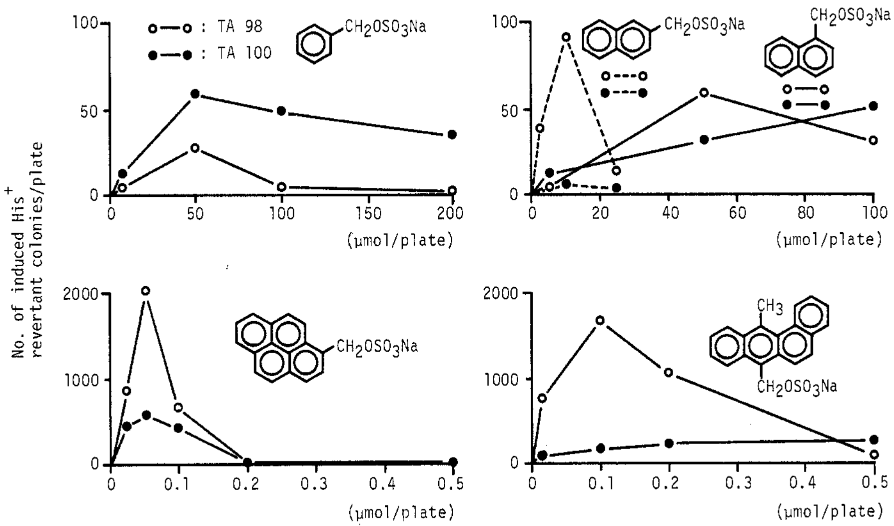

Fig. 2. Intrinsic mutagenicity of arylmethyl sulfates toward *Salmonella typhimurium* TA98 and TA100. Mutagenicity tests were carried out by the method of Ames *et al.* (*13*) in the absence of the hepatic preparation and fortifying agents.

and 228 (base) when analyzed on a gas-liquid chromatograph–mass spectrometer (Hitachi model RMU-7L). Under the conditions used, the methyl ester of 7-HMBA sulfate eluted at 8.9 minutes from a column packed with 1 percent OV-1 coated on Chromosorb W (60 to 80 mesh, 3 mm by 1 m), kept at 270°C, and developed with He at 30 ml/min. 7-HMBA sulfate (*15*) was synthesized and isolated as a sodium salt by treatment of 7-HMBA with chlorosulfonic acid in dry pyridine at room temperature, followed by neutralization with an aqueous NaOH solution. The synthetic specimen was spectroscopically and chromatographically identical with the mutagenic water-soluble metabolite in all respects. The rate of formation of 7-HMBA sulfate from 7-HMBA by the hepatic sulfotransferase-PAPS system was 0.50 nmole per milligram of protein per minute. 7-HMBA sulfate was also isolated and identified after DMBA was incubated with a 9000*g* supernatant fraction in the presence of both PAPS- and NADPH-generating systems. Thus a new mechanism for the metabolic activation of DMBA has been established, as depicted in Fig. 1.

The bacterial mutagenicity of 7-HMBA in the presence of the hepatic sulfotransferase-PAPS system was higher than that of DMBA or 7-HMBA in the presence of an NADPH-generating system and microsomes equivalent to the amount in the rat liver from which the soluble supernatant fraction was isolated. Thus DMBA and 7-HMBA (1.0 μmole per plate each in the 20-minute incubation media) induced 450 and 59 His$^+$ revertant colonies per plate in the TA98 strain, respectively. This suggests that the sulfate ester of 7-HMBA plays a much greater role in mutation than do the epoxides formed from DMBA or 7-HMBA by microsomes.

The participation of sulfate esters as ultimate forms in the metabolic activation of carcinogenic aromatic amines by rat liver was proposed and extensively studied by Miller *et al.* (*16*, *17*) and Weisburger *et al.* (*18*). The proposed activation mechanism is that *N*-monosubstituted aromatic amines such as 2-acetylaminofluorene (AAF) and *N*-methyl-4-aminoazobenzene (MAB) are oxidized by hepatic microsomal cytochrome P-450 to the corresponding *N*-hydroxy derivatives, which are then converted to sulfates by liver cytosolic sulfotransferase in the presence of PAPS (*17*). No direct evidence, however, has been obtained for the biological formation of sulfate esters of *N*-hydroxy aromatic amines as active metabolites. Thus the results reported here are, to our knowledge, the first direct evidence not only for the participation of the metabolically formed sulfate ester of a carcinogen in cell mutation, but also for the isolation and identification of a direct-acting mutagenic metabolite other than epoxides of carcinogenic hydrocarbons.

7-HMBA sulfate reacted at *p*H 7.4 with glutathione and ethyl mercaptan to produce the corresponding sulfides, and the reactions were accelerated by increasing the *p*H. This suggests that the sulfate may react with bacterial DNA bases as a 7-methylene carbonium ion of DMBA with loss of a sulfate anion (Fig. 1). The presumed role of the 7-methylene carbonium ion as ultimate reactant with DNA would be analogous to the mechanism of covalent binding of the carcinogens 7-bromomethyl-BA and 7-bromomethyl-12-methyl-BA to DNA (*19*).

Some other synthetic sulfate esters (*20*) of arylmethanols such as benzyl alcohol, 1- and 2-hydroxymethylnaphthalene, and 1-hydroxymethylpyrene were mutagenic toward *S. typhimurium* TA98 in increasing order (Fig. 2). Alkyl sulfates such as methyl, ethyl, *n*-propyl, and *n*-butyl sulfates were nonmutagenic under the same conditions.

Table 1. Mutagenicity of 7-HMBA toward *Salmonella typhimurium* TA98 in the presence of a rat liver cytosolic fraction fortified with a PAPS-generating system.

Testing system	His$^+$ revertant colonies per plate*
Control	39 (−)
Complete†	921 (882)
Minus Na_2SO_4	41 (2)
Minus ATP	42 (3)
Minus hepatic cytosol	39 (0)
Complete (boiled hepatic cytosol)	39 (0)
Complete plus *Helix pomatia* sulfatase (100 units)‡	46 (7)

*Values in parentheses were obtained by subtracting the mean number (39) of His$^+$ revertant colonies on three control plates from those on the other plates. The bacteria were kindly donated by B. N. Ames. †The complete system consisted of the following in a final volume of 1 ml of 0.1*M* Na_2HPO_4-KH_2PO_4 buffer, *p*H 7.4: 7-HMBA (1 m*M*) dissolved in dimethyl sulfoxide (0.1 ml), bacterial cells (10^9), dialyzed hepatic cytosol fraction (0.48 mg of protein, equivalent to 50 mg of liver, wet weight), Na_2SO_4 (5 m*M*), ATP (5 m*M*), $MgCl_2$ (3 m*M*), and EDTA (0.1 m*M*). The hepatic fraction was prepared by centrifugation (105,000*g*, 60 minutes) of the post-mitochondrial fraction from a three-volume homogenate of rat liver (90 to 110 g of body weight), followed by dialysis of the separated soluble supernatant fraction at 1°C for 20 hours against 2500 volumes of the phosphate buffer. ‡A Sigma type H-1 preparation was used.

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14. The methylation was carried out as follows. A residue obtained on evaporation of the solvent from the methanolic effluent was dissolved in 0.1*N* HCl. The free sulfate ester was immediately extracted with ethyl acetate after the saturation of the acidic solution with NaCl. Then an ethereal solution of diazomethane was added to the organic phase.
15. The sulfate was synthesized by reaction of 7-HMBA (5.0 mmole) with freshly redistilled chlorosulfonic acid (5.5 mmole) in anhydrous pyridine (20 ml) at room temperature. After 3 hours the mixture was titrated with an aqueous NaOH (11.5 mmole) solution. The neutralized mixture was diluted with ether (ten volumes) in three portions. The white precipitate that resulted was collected by filtration, washed thoroughly with ether, and suspended in ethanol (10 ml). The ethanol-insoluble inorganic salts were removed by filtration, and the filtrate was concentrated to dryness in vacuo at 20°C. The residue was redissolved in ethanol (5 ml), and the solution filtered through. The sodium salt of the sulfate ester was precipitated from the filtrate by the addition of ether (ten volumes), collected by filtration, washed with ether, and dried in vacuo. Overall yield of the sulfate was 70 percent. Spectroscopic results for 7-HMBA sulfate (Na) are: IR ν_{max}^{KBr} cm^{-1} = 3040, 2949, 2860, 1638, 1495, 1269, 1230, and 1066; UV λ_{max}^{EtOH} nm (ϵ) = 212 (24,503), 264 (18,197), 272 (22,909), 282 (34,674), 293 (34,674), 340 (7,692), 356 (10,440), and 374 (8,791); NMR δ_{ppm} in CD_3OD = 3.16 (methyl H, *s*), 5.43 (methylene H, *d*, *J* = 7,7), and 7.20 to 8.45 (aromatic H, *m*).
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20. The sulfates were synthesized from the corresponding arylmethanols by the method described for 7-HMBA sulfate (*14*). Benzyl sulfate (Na): IR ν_{max}^{KBr} cm^{-1} = 3050, 3012, 2875, 1497, 1465, 1250, 1201, and 1080; UV λ_{max}^{EtOH} nm (ϵ) = 212 (2686) and 257 (124); NMR δ_{ppm} in CD_3OD = 5.06 (methylene H, *s*) and 7.43 (aromatic H, *s*). 1-Hydroxymethylnaphthalene sulfate (Na): IR ν_{max}^{KBr} cm^{-1} = 3000, 2850, 1592, 1506, 1251, and 1210; UV λ_{max}^{EtOH} nm (ϵ) = 224 (73666), 270 (4613), 280 (5390), and 290 (4194); NMR δ_{ppm} in CD_3OD = 5.53 (methylene H, *d*, *J* = 7.4) and 7.53 to 8.43 (aromatic H, *m*). 2-Hydroxymethylnaphthalene sulfate (Na): IR ν_{max}^{KBr} cm^{-1} = 3050, 2850, 1595, 1500, 1245, and 1213; UV λ_{max}^{EtOH} nm (ϵ) = 230 (73714), 275 (4629), 282 (5411), and 298 (4208); NMR δ_{ppm} in CD_3OD = 5.55 (methylene H, *d*, *J* = 7.6) and 7.60 to 8.54 (aromatic H, *m*). 1-Hydroxymethylpyrene sulfate (Na): IR ν_{max}^{KBr} cm^{-1} = 3020, 2900, 1606, 1584, 1450, 1265, 1235, and 1070; UV λ_{max}^{EtOH} nm (ϵ) = 233 (8901), 241 (14256), 264 (5271), 274 (9541), 311 (2503), 326 (6120), and 342 (9179); NMR δ_{ppm} in CD_3OD = 5.40 (methylene H, *d*, *J* = 7.3) and 7.65 to 8.35 (aromatic, H, *m*).

9 June 1981; revised 12 August 1981

Expression of Bovine Leukemia Virus Genome Is Blocked by a Nonimmunoglobulin Protein in Plasma from Infected Cattle

Abstract. *Plasma of cattle infected with bovine leukemia virus contains a soluble factor that blocks the expression of the viral genome in cultured lymphocytes. The blocking factor is not present in plasma of bovine leukemia virus–free cattle or of cattle infected with common bovine viruses. Blocking of bovine leukemia virus expression by the plasma factor is reversible, and seems to be mediated by a nonimmunoglobulin protein molecule.*

Bovine leukemia virus (BLV) is regarded as the causative agent of the adult or enzootic form of bovine leukemia, which is the most common neoplasia of cattle (*1*). In naturally infected cattle, BLV has been detected only in the lymphocytes (*2*). However, regardless of whether the BLV-infected lymphocytes are neoplastic or not, they produce virus particles and express the major internal (p25) and glycoprotein (gp51) virion antigens only after cultivation in vitro for a few hours (*2–4*). Molecular hybridization studies in which a BLV complementary DNA probe was used failed to detect BLV-specific RNA in infected lymphocytes before cultivation in vitro. In contrast, significant amounts of viral RNA were detected in the same cells after cultivation (*2*).

These results suggest that, in vivo, the BLV genome is repressed at the transcriptional level. BLV is the only known virus responsible for spontaneous leukemia, which is present in the infected cells in a repressed state. It is likely that virus-neutralizing antibodies, which are present in virtually all BLV-infected cattle (*5*, *6*), interfere with virus production in the infected lymphocytes. However, it is less clear how viral antibodies block the expression of the BLV genome. The present study indicates that expression of the BLV genome is blocked by a protein factor present in the plasma of BLV-infected cattle.

The amount of p25 produced by BLV-infected lymphocytes during short-term cultivation in vitro is ten times higher than that of other virion proteins (*7*). Therefore, in this study the synthesis of p25 was used as the indicator of BLV genome expression.

Lymphocytes were obtained without hypotonic shock from the buffy coat of the blood of BLV-infected cattle, cultured for 24 hours under a variety of conditions, and tested for the presence of BLV p25 by competitive radioimmunoassay (*8*). As shown in Table 1, no BLV p25 was detected in infected lymphocytes cultured with 100 percent autologous plasma. In contrast, p25 was readily detected when these cells were cultured in 100 percent autologous serum or in 100 percent plasma or serum from a BLV-free cow. Recovery of viable cells was greater than 70 percent in all cultures. The absence of a detectable blocking effect in the serum of BLV-infected cattle indicates that the blocking factor is not an antibody. Further evidence supporting this conclusion was obtained in an experiment in which BLV-infected lymphocytes were cultured in the presence of BLV-negative bovine plasma supplemented with serum gamma globulin (30 mg/ml) from a BLV-infected animal. Even though this concentration of gamma globulin is equivalent to that normally present in cattle serum, no blocking of p25 expression was observed (Table 1).

The blocking factor was found in the plasma of nine of ten BLV-infected cattle. Expression was blocked by either autologous or homologous plasma from BLV-infected cattle. The blocking effect was also observed when the infected lymphocytes were cultured for 48 hours rather than 24 hours. No blocking was

Table 1. Expression of BLV p25 antigen in cultured lymphocytes from a BLV-infected cow. Lymphocytes were isolated (without hypotonic shock) from the buffy coat of heparinized peripheral blood and cultured at 37°C for 24 hours at a density of 3×10^6 cells per milliliter in 20 ml of 100 percent plasma or serum. Following incubation, the cells were harvested, washed twice, disrupted, and tested by competitive radioimmunoassay for BLV p25 (*8*).

Additions to BLV-infected lymphocytes in culture	BLV p25 in lymphocytes (nanograms per milliliter of cell extract)
100 percent autologous plasma	< 0.3
100 percent autologous serum	7
100 percent BLV-negative plasma	6
100 percent BLV-negative serum	7
100 percent BLV-negative plasma mixed with gamma globulin* from BLV-infected cow	6

*The titer of the gamma globulin at a concentration of 1 mg/ml was 1:1000 in the radioimmunoassay.

0036-8075/82/0122-0405$01.00/0