Hepatic Metabolism of Methyl Anthranilate and Methyl N-Methylantranilate as Food Flavoring Agents in Relation to Allergenicity in the Guinea Pig

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To assess the safety of the food flavoring agents, methyl anthranilate (MA) and methyl N-methylantranilate (MMMA), their relationships with metabolism and allergenicity were examined in guinea pigs. Both MA and MMMA were hydrolyzed to anthranilic acid (AA) and N-methylantranilic acid (N-methylAA), respectively, by guinea pig liver microsomes. These hydrolytic activities at 1000 µM were 320 and 35 nmol/min/mg protein, respectively. Moreover, MMMA was N-demethylated to MA by the liver microsomes; the oxidative activity at 1000 µM of MMMA was 2.8 nmol/min/mg protein. The N-demethylase activity for N-methylAA at 1000 µM in the liver microsomes was 3.9 nmol/min/mg protein. Kinetic analysis indicated that the V_{max}/K_{m} values of MA and MMMA hydrolyses were 15- and 7.4-fold greater in guinea pig liver microsomes than in the cytosol, respectively, suggesting that these hydrolytic activities were predominantly localized in the microsomes. Liver microsomal activities for the hydrolysis of these flavoring esters were markedly inhibited by diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and bis(p-nitrophenyl)phosphate but not by physostigmine. These hydrolytic activities were suppressed by aspirin, a substrate of carboxylesterase, in a concentration-dependent manner. The N-demethylations of MMMA and N-methylAA were inhibited by SKF 525-A, a nonselective inhibitor of cytochrome P450. At the same time as the metabolic study described above, skin reactions in guinea pigs were investigated using MA, MMMA, N-methylAA, and AA. All compounds examined elicited positive skin reactions, although MMMA and AA exhibited relatively greater sensitizing properties. These results may provide useful information about metabolism in the toxicologic evaluations of MA and MMMA.

Key words —– methyl anthranilate, methyl N-methylantranilate, anthranilic acid, carboxylesterase, cytochrome P450, skin reaction

INTRODUCTION

Food flavoring agents are classified into two groups, synthetic and natural compounds. Currently, 104 synthetic flavoring agents are legally approved for use in Japan. Synthetic flavoring ingredients are volatile compounds with molecular weight of less than 300 and consist of esters, aldehydes, alcohols, ketones, carboxylic acids, aryl phenols, and lactones. Most people, from adults to children, consume foods and beverages with food flavoring agents daily. However, synthetic flavoring ingredients are not always harmless although they are used in low levels in food. We reported previously that flavoring aldehydes such as p-anisaldehyde and n-octanal elicited skin reactions in guinea pigs. Thus it is very important to assess the safety of food flavoring agents in relation to their toxicokinetics.

Methyl anthranilate (MA) and methyl N-methylantranilate (MMMA) are flavoring esters (Fig. 1) added to many foods such as ice cream, candy, and chewing gum. Previous studies have shown that MA is rapidly hydrolyzed to anthranilic acid (AA) by homogenates from rat and pig livers.
Grundschober reported that MNMA was hydrolyzed to N-methylanthranilic acid (N-methylAA) by pig liver homogenate. Furthermore, it has been demonstrated that N-methylAA and AA at the ratio of approximately 20 : 1 are detected in urine 24 hr after the oral administration of MNMA to rats. These findings suggest that MNMA is metabolized to AA via N-methylAA and/or MA as intermediate metabolites (Fig. 1). Toxicologically, MA has been reported to produce moderate irritation in rabbit skin for 24 hr under occlusion. However, the hepatic metabolism and allergenicity of MA, MNMA, and their metabolites have not been examined extensively. In the present study, we investigated the in vitro metabolism of MA and MNMA to AA and skin reactions caused by MA, MNMA, N-methylAA, and AA.

MATERIALS AND METHODS

Materials — MA, MNMA, N-methylAA, AA, methyl p-methoxybenzoate, diisopropyl fluorophosphosphate (DFP), and aspirin were obtained from Wako Pure Chemical Industries (Osaka, Japan). Phenylmethylsulfonyl fluoride (PMSF), bis(p-nitrophenyl)phosphate (BNPP), SKF 525-A, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NADP and glucose-6-phosphate were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). Other chemicals and solvents used were of the highest quality commercially available.

Animals — Male Hartley guinea pigs were purchased from Sankyo Laboratories (Toyama, Japan). The animals were given water and food ad libitum. Preparation of Liver Microsomes and Cytosol —— Male Hartley strain guinea pigs (8 weeks old) were anesthetized with ether and killed by exsanguination. Liver microsomes and cytosol were prepared as reported previously. The protein concentration was estimated using the method of Lowry et al. with bovine serum albumin as a standard. Enzyme Assays —— The hydrolytic activity for MA was determined as described below, unless otherwise stated. MA was incubated with guinea pig liver microsomes or cytosol (100 µg protein) in sodium-potassium phosphate buffer 38 mM (pH 7.4) in a final volume of 260 µl. The mixture was incubated at 37°C for 10 min, and the reaction was terminated by the addition of acetonitrile 200 µl containing methyl p-methoxybenzoate (0.20 µg) as an internal standard. After the removal of protein by centrifugation, 50 µl of the supernatant was injected onto a high-performance liquid chromatography (HPLC) system (LaChrom L-7000 series; Hitachi, Tokyo, Japan) equipped with an Inertsil ODS-2 column (4.6 × 250 mm, 5 µm, GL Sciences, Tokyo, Japan). The mobile phase was ammonium dihydrogenphosphate 30 mM containing 40% acetonitrile. Elution was performed at a flow rate of 0.7 ml/min. The formation of AA was monitored at a wavelength of 255 nm using a LaChrom UV detector L-7400 (Hitachi). Peak areas obtained were integrated with a D-2500 chromato-integrator (Hitachi).

The hydrolytic activity for MNMA was determined as described below, unless otherwise stated. MNMA was incubated with guinea pig liver microsomes (5 µg protein) or cytosol (50 µg protein) in sodium-potassium phosphate buffer 38 mM (pH 7.4) in a final volume of 260 µl. Other methods were the same as described for MA hydrolysis, except for the incubation time of 5 min. The formation of N-methylAA was monitored at a wavelength of 255 nm. The oxidative activity for MNMA was determined as described below. MNMA (1000 µM) was incubated with guinea pig liver microsomes (500 µg protein), an NADPH-generating system (NADP 0.5 mM, glucose 6-phosphate 10 mM, MgCl₂ 10 mM, and 1 unit of glucose-6-phosphate dehydrogenase), and sodium-potassium phosphate buffer 100 mM (pH 7.4) in a final volume of 1 ml. The mixture was incubated at 37°C for 5 min, and the reaction was terminated by the addition of diethyl ether 5 ml. After the addition of methyl p-methoxy-
benzoate (0.20 µg) as an internal standard, the mixture was extracted with diethyl ether and centrifuged at 800 × g for 5 min. The organic layer was transferred to another tube and the solvent was evaporated. The residue was dissolved in 200 µl of ammonium dihydrogenphosphate 30 mM containing 40% acetonitrile and subjected to HPLC. The HPLC conditions were the same as described for MNMA hydrolysis, except for monitoring the formation of MA at a wavelength of 255 nm.

The oxidative activity for N-methylAA was determined as described below. N-MethylAA (1000 µM) was incubated with guinea pig liver microsomes (500 µg protein), an NADPH-generating system, and sodium-potassium phosphate buffer 100 mM (pH 7.4) in a final volume of 1 ml. Other methods were the same as described for MNMA oxidation, except for the incubation time of 10 min. The formation of AA was monitored at a wavelength of 255 nm.

To determine the kinetic parameters for the hydrolysis of MA and MNMA, liver microsomes and cytosol from guinea pig were incubated with MA (40 to 2500 and 40 to 1600 µM, respectively) or MNMA (100 to 750 and 20 to 500 µM, respectively) under the same conditions described above. Data points were fitted to the Michaelis-Menten equation using nonlinear least-square regression analysis with Origin 7.5J software (OriginLab, Northampton, MA, U.S.A.).

**Effects of Various Inhibitors on Hydrolysis and Oxidation of AA Derivatives** —— For hydrolysis, guinea pig liver microsomes were preincubated with DFP (0.1 and 1 µM), PMSF (1 and 10 µM), BNPP (0.1, 1 and 10 µM), and physostigmine (100 µM) at 37°C for 5 min. Subsequently, the mixture was incubated with MA or MNMA (250 µM) in the same manner as described for the enzyme assays. Since aspirin was very rapidly hydrolyzed by guinea pig liver microsomes, aspirin (250, 500, and 1000 µM) was added to the reaction mixture just before initiating the incubation. After preincubation with guinea pig liver microsomes and SKF 525-A (500 µM) at 37°C for 5 min, the mixture for the oxidations was incubated with MNMA or N-methylAA (1000 µM). Other methods were the same as described for MNMA and N-methylAA oxidation. All inhibitors except for SKF 525-A in water were dissolved in dimethyl sulfoxide and added to the incubation mixture at a final dimethyl sulfoxide concentration of 1%.

**Sensitization and Skin Reaction** —— Male Hartley strain guinea pigs (5 weeks old) were sensitized by the method described previously, with a slight modification. Each group consisted of three to five animals. All test compounds (MA, MNMA, N-methylAA, and AA) were dissolved in corn oil. For sensitization, the animals were injected with 0.1 ml of the test compound solution (1.0%) intracutaneously on the abdomen for 7 days. Control animals received 0.1 ml of corn oil intracutaneously instead of the test compound solution. Three weeks later, elicitation experiments were carried out with an injection of 0.1 ml of 0.25% to 1.0% test compound solution or corn oil as the control. The abdomen of each animal was depilated with a hair remover (EBA cream, Mitsubishi Pharma Corporation, Osaka, Japan) prior to the elicitation of a skin reaction. Results of skin reactions are expressed as the diameter of erythema 24 hr after the injection of test compounds.

**RESULTS**

**Metabolism of MA and MNMA by Microsomes and Cytosol of Guinea Pig Liver**

Representative chromatograms are shown in Fig. 2. MA and MNMA were hydrolyzed by guinea pig liver microsomes to produce AA and N-methylAA, respectively (Fig. 2B and 2C). The formation of these metabolites did not require an NADPH-generating system (data not shown). MNMA and N-methylAA were oxidized by liver microsomes to MA and AA, respectively, in the presence of the NADPH-generating system (Table 1). However, when MNMA was used as a substrate, further metabolism of N-methylAA to AA was not seen under the conditions used in this study (data not shown). Table 1 summarizes individual activities of hydrolysis and oxidation of MA, MNMA, and N-methylAA by guinea pig liver microsomes and cytosol. When MA was used as a substrate, the liver microsomes showed a 33-fold greater hydrolytic activity compared with that of the cytosol. The hydrolytic activity for MNMA was 18-fold greater in the liver microsomes than in the cytosol. Furthermore, the liver microsomal activity of MNMA hydrolysis was approximately 13-fold greater than that of MNMA N-demethylation. The oxidation of N-methylAA in liver microsomes was 1.4-fold greater than that of MNMA. Since the protein concentrations of the liver microsomes and cytosol were 27
and 89 mg/g liver, respectively, the total hydrolytic activity for MA in guinea pig liver was 90-fold greater than oxidative activities for MNMA and N-methylAA; the total hydrolytic activity for MNMA was 10-fold greater as compared with MNMA and N-methylAA oxidative activities.

Kinetic analysis of the hydrolysis of MA and MNMA was performed with microsomes and cytosol of guinea pig liver. All hydrolytic activities were saturated within the substrate concentrations examined (Fig. 3). Table 2 summarizes the kinetic parameters for the hydrolysis of MA and MNMA catalyzed by guinea pig liver microsomes and cytosol. The $V_{\text{max}}$ values of these hydrolytic reactions were approximately 30-fold greater in the liver microsomes than in the liver cytosol. In addition, the $K_{m}$ values of MA and MNMA hydrolysates by liver microsomes were 1.8- and 4.3-fold greater than those by the cytosol, respectively. Thus the $V_{\text{max}}/K_{m}$ values, reflecting intrinsic clearance, for the hydrolysis of these flavoring esters were 15- and 7.4-fold greater in the microsomes than in the cytosol, respectively.

**Characterization of Enzymes Involved in Hydrolysis and Oxidation of MA, MNMA, and N-MethylAA in Guinea Pig Liver Microsomes Using Chemical Inhibitors**

To characterize the esterases responsible for the hydrolysis of MA and MNMA, we investigated effects of esterase inhibitors on these hydrolytic reactions. The hydrolytic activity for MA in liver microsomes from guinea pig was markedly inhib-
Table 2. Kinetic Parameters for the Hydrolysis of MA and MNMA in Liver Microsomes and Cytosol from Guinea Pig

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme source</th>
<th>$V_{\text{max}}$ (nmol/min/mg protein)</th>
<th>$K_m$ (µM)</th>
<th>$V_{\text{max}}/K_m$ (µl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>Microsomes</td>
<td>350 ± 9</td>
<td>210 ± 24</td>
<td>1700 ± 200</td>
</tr>
<tr>
<td></td>
<td>Cytosol</td>
<td>13 ± 1</td>
<td>120 ± 15</td>
<td>110 ± 16</td>
</tr>
<tr>
<td>MNMA</td>
<td>Microsomes</td>
<td>47 ± 5</td>
<td>280 ± 78</td>
<td>170 ± 50</td>
</tr>
<tr>
<td></td>
<td>Cytosol</td>
<td>1.5 ± 0.1</td>
<td>65 ± 10</td>
<td>23 ± 4</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± S.E. of kinetic parameters.

Table 3. Effects of Various Esterase Inhibitors on Hydrolysis of MA and MNMA in Guinea Pig Liver Microsomes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µM)</th>
<th>Residual activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MA</td>
<td>MNMA</td>
</tr>
<tr>
<td>DFP</td>
<td>0.1</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>PMSF</td>
<td>1</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>BNPP</td>
<td>0.1</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>100</td>
<td>94</td>
</tr>
</tbody>
</table>

a) Control activities for MA and MNMA (250 µM) without inhibitors were 210 and 33 nmol/min/mg protein, respectively. All determinations were performed in duplicate.
ited by DFP and PMSF, inhibitors of serine hydrolases such as carboxylesterase and cholinesterase (Table 3). This activity was also effectively suppressed by BNPP, a selective inhibitor of carboxylesterase, whereas the hydrolysis was not inhibited by physostigmine, a cholinesterase inhibitor. Similarly, the hydrolytic activity for MNMA was inhibited by DFP, PMSF, and BNPP but not by physostigmine. In addition, aspirin, a carboxylesterase substrate, caused a concentration-dependent inhibition of hydrolytic activities for MA and MNMA (Fig. 4). Both hydrolytic activities were inhibited to approximately 20% of the control activities when aspirin at a concentration of 1000 µM was used as an inhibitor.

To clarify the involvement of P450 in the oxidation of MNMA and -methylAA, an inhibition study was carried out with SKF 525-A, a nonselective inhibitor of P450 (Fig. 5). This inhibitor (500 µM) decreased the liver microsomal activities for MNMA and -methylAA up to 58 and 8.7%, respectively.

Skin Reaction in Guinea Pigs Due to AA Derivatives

The skin reaction in guinea pigs was investigated with MA, MNMA, -methylAA, and AA. These test compounds induced positive skin reactions in all animals examined. Figure 6 indicates the dependence of the erythema size on concentrations of the AA derivatives injected. The erythema induced by MA (n = 4) reached a maximum at a concentration of 0.25%. However, a concentration-dependent induction was not seen in the skin reaction due to MA. The erythema elicited by MNMA (n = 3), -methylAA (n = 5), and AA (n = 4) increased as the concentration of these compounds increased. Among the test compounds investigated, MNMA and AA exhibited relatively greater sensitizing properties.

DISCUSSION

The results of present study demonstrated that MA and MNMA were hydrolyzed and/or -demethylated by guinea pig liver microsomes and cytosol to form AA. MNMA was mainly hydrolyzed to -methylAA by liver microsomes, in addition to oxidation resulting in the formation of MA. -MethylAA was metabolized to AA by liver microsomes in the presence of an NADPH-generating system. Moreover, MA was efficiently hydrolyzed by the liver microsomes and cytosol to produce AA. These results suggest that AA is formed from MNMA.
in the liver via both pathways of $N$-methylAA formation and MA formation.

Kinetic analysis of the hydrolysis of MA and MNMA by the liver microsomes indicated that higher $V_{\text{max}}/K_m$ values were seen in guinea pig liver microsomes as compared with the liver cytosol. Intrinsic clearance of the hydrolysis of MA and MNMA by the liver microsomes was 4.7- and 2.2-fold greater than that by the cytosol, respectively, when protein concentrations of the liver microsomes (27 mg/g liver) and cytosol (89 mg/g liver) were taken into account. This result suggests that liver microsomes contribute greatly to the hydrolysis of these flavoring esters in comparison to the cytosol. The hydrolysis of MA by guinea pig liver microsomes and cytosol showed higher $V_{\text{max}}/K_m$ values as compared with that of MNMA by the corresponding enzyme sources. The $N$-methyl group appears to influence the ability of liver esterases to hydrolyze MA and MNMA.

Inhibition of the hydrolysis of MA and MNMA by DFP and PMSF in guinea pig liver microsomes indicates that these reactions are catalyzed by serine hydrolases containing carboxylesterase and cholinesterase. Furthermore, the inhibitory effect of BNPP suggests that carboxylesterase is responsible for these hydrolytic reactions in guinea pig liver microsomes. In contrast, cholinesterase appears not to be mainly involved in these reactions because physostigmine did not show inhibitory effects. The inhibition study results using aspirin suggest that an aspirin hydrolase may be at least partly responsible for the hydrolysis of MA and MNMA in liver microsomes of guinea pig. White and Hope reported that aspirin is specifically hydrolyzed by a single microsomal esterase of guinea pig liver. The aspirin hydrolase partially purified from guinea pig liver microsomes is monomeric and has a molecular weight of approximately 57,100, although the isoelectric point has not been determined. This enzyme shows the characteristics of carboxylesterase, i.e., it is sensitive to inhibition by BNPP. Although a classification of the carboxylesterase superfamily has been proposed by Satoh and Hosokawa, aspirin hydrolase has not been classified into any family due to a lack of information on the nucleotide sequence in the coding region of the corresponding gene. In humans, aspirin is known to be specifically hydrolyzed by carboxylesterase 2. This finding suggests that the aspirin hydrolase of guinea pig may belong to a carboxylesterase 2 family.

The inhibitory effects of SKF 525-A on $N$-demethylations of MNMA and $N$-methylAA by guinea pig liver microsomes suggest the involvement of P450 in these oxidations. P450 is known to catalyze the oxidation of a wide variety of endogenous and exogenous compounds, including drugs and other xenobiotic chemicals. P450 comprises a superfamily containing numerous isoenzymes with distinct substrate specificities. However, the P450 isoenzyme(s) responsible for the $N$-demethylation of MNMA and $N$-methylAA remains to be identified.

In the present study, MA and MNMA produced positive skin reactions in guinea pigs, although MA...
did not show a concentration-dependent induction. It has been reported that MA elicits a skin reaction in rabbits. This finding and our results suggest that MA and MNMA may exhibit allergic properties. Interestingly, AA, a metabolite of MA and MNMA, also produced skin reactions in guinea pigs in this study. A previous study showed that AA deteriorates adjuvant-induced arthritis in rats and enhances antibody production in mice immunized with sheep erythrocytes, indicating that AA has immunopotentiating properties. These observations suggest that AA partly contributes to sensitization to MA and MNMA. Further studies on the allergenicity of MA and MNMA when ones consume food and beverages containing these flavoring agents. However, the mechanism by which AA elicits the skin reaction is unclear at present, as are the mechanisms of the skin reactions induced by MA and MNMA.

The hydrolysis of methyl esters such as MA and MNMA results in the production of methanol, which is further metabolized to formaldehyde by alcohol dehydrogenase and catalase. Moreover, the N-dealkylation of N-methyl compounds including MNMA leads to the formation of formaldehyde. It has been reported that the injection of formaldehyde in guinea pig footpads induces a skin reaction and produces antibodies to this allergen. These findings imply that formaldehyde formed from MA and MNMA could contribute in part to sensitization to these flavoring agents. However, there is no direct evidence that formaldehyde is involved in the skin reactions elicited by MA and MNMA. Further studies on the allergenicity of MA, MNMA, and their metabolites are therefore needed.

The present study conclusively characterized the metabolic pathway of MA and MNMA to AA in the guinea pig liver and relatively greater sensitizing potential of MNMA and AA. In addition, our results suggest that guinea pig liver microsomal carboxylesterases, at least partly aspirin hydrolase, are responsible for the hydrolysis of MA and MNMA, and that P450 catalyzes the N-demethylation of MNMA and N-methylAA in liver microsomes. These results may provide useful information for metabolic studies of the toxicologic evaluation of MA and MNMA.

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REFERENCES


