Metabolic Activation and Inactivation of Metanil Yellow and Orange II in the Salmonella typhimurium his− Reversion Assay

P. B. RASTOGI and R. E. LEVIN*

Department of Food Science and Nutrition, Massachusetts Agricultural Experiment Station, University of Massachusetts, Amherst, Massachusetts 01003, USA

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ABSTRACT

Among a variety of experimental protocols used, the combined use of 0.5% dextrose in bottom agar and 1 μmol of flavin mononucleotide (FMN) in preincubation mixtures without fraction S9 mix resulted in the highest level of induced Salmonella typhimurium his− reversions with both dyes metanil yellow and orange II with strain TA100. Strain TA98 yielded notably lower levels of reversions under the same conditions. The presence of uninduced hamster liver S9 fraction resulted in a weak mutagenic response while Aroclor 1254-induced rat liver S9 fraction resulted in the complete absence of mutagenicity with both strains and with both dyes.

Key words: Metanil yellow, orange II, mutagenicity, Salmonella

The use of the dyes metanil yellow and orange II as food colorants is not permitted in India. However, a detailed analysis of colored food samples in India (11) revealed that as many as 29 and 11% of the total colored food samples examined contained metanil yellow and orange II respectively. These dyes have been used in bakery products, beverages, ice-candy, confectionary, dairy products, sausages, pet foods, snacks, and multicolored medicinal tablets. Both dyes also find application in the dyeing of cotton, wool, paper, colored soaps, etc. (10). Genotoxicity, mutagenicity, and carcinogenicity have been reported for both dyes in mammalian assays (16, 17, 20). In contrast, nonmutagenicity of both dyes in a variety of microbial assays has been reported (6, 12, 14, 15, 22). A weak mutagenic response of orange II with S. typhimurium TA1538 has also been reported (7).

Most studies on the microbial mutagenicity of food colorants have involved those pigments used primarily in the United States and have been reviewed (8). There is little reference to food dyes used elsewhere in the world or to dyes other than food colorants (2). The purpose of the present study was to determine the optimum microbial assay system for detection of mutagenicity by these two dyes with the S. typhimurium his− system.

MATERIALS AND METHODS

Cultures

Salmonella typhimurium tester strains TA98 for detection of frameshift mutations and TA100 for detection of base-pair substitu-

tion mutations were kindly provided by B. N. Ames and have been previously characterized (1). Prior to use, both cultures were routinely tested for his−, rfa, amp+ (presence of the pKM101 plasmid) phenotypes, the rate of spontaneous reversion, and their response to the diagnostic mutagens benzo[a]pyrene and 2,4,7-trinitrofluorenone (2,4,7-TNF).

Chemicals

Metanil yellow (sodium salt of m-[(p-anilinophenyl)azo]benzenesulfonic acid), orange II (sodium salt of 4-[[2-hydroxy-1-naphthalenyl]azo]-benzenesulfonic acid), glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate (NADP), NADPH, and reduced NAD (NADH), flavin mononucleotide (FMN), benzo[a]pyrene and adenosine triphosphate (ATP) were purchased from Sigma Chemical Co. G-6-P dehydrogenase was purchased from Calbiochem, riboflavin from General Biochemi-

* Author for correspondence. Tel: 413-545-0187.
TABLE 1. Composition of fraction S9 mix and the various protocols used

<table>
<thead>
<tr>
<th>Variables</th>
<th>I Ames assay</th>
<th>II Ames assay with preinc.</th>
<th>III Riboflavin assay</th>
<th>IV Yahagi's assay</th>
<th>V Azo dye protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9 mix (μmol/ml):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Na)PO₄ buffer, pH 7.4</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>KCl</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>NADP</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NADH</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>NADPH</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>G-6-P</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>G-6-P dehyd. (units/ml)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FMN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S9 mix prepared in:</td>
<td>0.15 M KCl</td>
<td>0.15 M KCl</td>
<td>0.15 M KCl</td>
<td>0.15 M KCl</td>
<td>0.1 M (Na)PO₄, pH 7.4</td>
</tr>
<tr>
<td>Preincubation</td>
<td>None</td>
<td>37°C, 20 min</td>
<td>37°C, 20 min</td>
<td>37°C, 20 min</td>
<td>30°C, 30 min</td>
</tr>
<tr>
<td>% glucose in bottom agar</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a Yahagi et al. (26, 27).

RESULTS

Strain TA98

Both dyes failed to yield mutagenesis with the standard Ames assay (1) using strain TA98 with and without Aroclor 1254-induced rat liver S9 (Table 2).

TABLE 2. Mutagenicity of metanil yellow and orange II with S. typhimurium TA98

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Dose (μmol)</th>
<th>0.01</th>
<th>0.03</th>
<th>0.10</th>
<th>0.30</th>
<th>1.00</th>
<th>3.00</th>
<th>0.01</th>
<th>0.03</th>
<th>0.10</th>
<th>0.30</th>
<th>1.00</th>
<th>3.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metanil yellow</td>
<td>29 ± 2</td>
<td>55 ± 7</td>
<td>37 ± 1</td>
<td>44 ± 5</td>
<td>39 ± 6</td>
<td>55 ± 1</td>
<td>—</td>
<td>—</td>
<td>38 ± 2</td>
<td>66 ± 5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Orange II</td>
<td>35 ± 4</td>
<td>48 ± 5</td>
<td>43 ± 4</td>
<td>50 ± 2</td>
<td>49 ± 7</td>
<td>45 ± 3</td>
<td>—</td>
<td>—</td>
<td>65 ± 5</td>
<td>85 ± 11</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Benz(a)pyrene</td>
<td>46 ± 1</td>
<td>277 ± 51</td>
<td>37 ± 5</td>
<td>231 ± 19</td>
<td>40 ± 7</td>
<td>192 ± 14</td>
<td>48 ± 1</td>
<td>212 ± 14</td>
<td>54 ± 2</td>
<td>305 ± 55</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2,4,7 TNP</td>
<td>8352 ± 84</td>
<td>544 ± 24</td>
<td>7912 ± 348</td>
<td>473 ± 6</td>
<td>8992 ± 332</td>
<td>577 ± 51</td>
<td>7514 ± 314</td>
<td>560 ± 32</td>
<td>8049 ± 157</td>
<td>542 ± 21</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>None</td>
<td>42 ± 4</td>
<td>51 ± 6</td>
<td>36 ± 4</td>
<td>37 ± 5</td>
<td>35 ± 6</td>
<td>49 ± 2</td>
<td>46 ± 1</td>
<td>73 ± 5</td>
<td>45 ± 3</td>
<td>62 ± 10</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a Standard Ames plating assay without preincubation. With and without 0.5 ml Aroclor 1254-induced rat liver S9 mix (1).

b Same as a with preincubation for 20 min at 37°C.

c Same as b plus the protocol of Sugimura et al. (21) with preincubation for 20 min at 37°C.

Procedure of Yahagi et al. (26, 27) with preincubation for 20 min at 37°C. With and without 0.5 ml Aroclor 1254-induced rat liver S9 mix.

"Complete azo dye protocol" of Prival and Mitchell (19) with and without uninduced hamster liver S9 mix with preincubation for 30 min at 30°C.
TABLE 3. Mutagenicity of metanil yellow and orange II with *S. typhimurium* TA100

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Chemical (µmoles)</th>
<th>Dose</th>
<th>Ia</th>
<th>Ib</th>
<th>Ic</th>
<th>Ic</th>
<th>Vd</th>
<th>Ve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
<td>+S9</td>
<td>-S9</td>
</tr>
<tr>
<td>Metanil yellow</td>
<td>0.01</td>
<td>136 ± 2</td>
<td>142 ± 15</td>
<td>124 ± 3</td>
<td>121 ± 7</td>
<td>120 ± 14</td>
<td>120 ± 1</td>
<td>145 ± 14</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>134 ± 4</td>
<td>141 ± 19</td>
<td>114 ± 2</td>
<td>125 ± 11</td>
<td>139 ± 1</td>
<td>128 ± 2</td>
<td>133 ± 8</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>147 ± 4</td>
<td>143 ± 10</td>
<td>184 ± 2</td>
<td>134 ± 4</td>
<td>121 ± 3</td>
<td>123 ± 2</td>
<td>162 ± 5</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>148 ± 9</td>
<td>141 ± 7</td>
<td>237 ± 17</td>
<td>124 ± 3</td>
<td>122 ± 3</td>
<td>125 ± 6</td>
<td>167 ± 12</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>150 ± 2</td>
<td>162 ± 17</td>
<td>54 ± 28</td>
<td>80 ± 31</td>
<td>38 ± 1</td>
<td>114 ± 2</td>
<td>152 ± 3</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>138 ± 4</td>
<td>174 ± 6</td>
<td>78 ± 0</td>
<td>21 ± 6</td>
<td>25 ± 1</td>
<td>87 ± 29</td>
<td>137 ± 22</td>
</tr>
<tr>
<td>Orange II</td>
<td>0.01</td>
<td>149 ± 1</td>
<td>158 ± 14</td>
<td>125 ± 7</td>
<td>124 ± 3</td>
<td>127 ± 9</td>
<td>119 ± 1</td>
<td>148 ± 33</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>134 ± 4</td>
<td>139 ± 9</td>
<td>134 ± 3</td>
<td>125 ± 6</td>
<td>126 ± 5</td>
<td>124 ± 7</td>
<td>149 ± 9</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>145 ± 3</td>
<td>159 ± 7</td>
<td>267 ± 20</td>
<td>126 ± 5</td>
<td>127 ± 3</td>
<td>129 ± 12</td>
<td>195 ± 26</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>144 ± 10</td>
<td>142 ± 16</td>
<td>244 ± 10</td>
<td>125 ± 6</td>
<td>125 ± 7</td>
<td>152 ± 9</td>
<td>125 ± 6</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>144 ± 2</td>
<td>146 ± 12</td>
<td>139 ± 5</td>
<td>133 ± 7</td>
<td>114 ± 2</td>
<td>98 ± 2</td>
<td>133 ± 3</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>134 ± 4</td>
<td>154 ± 26</td>
<td>127 ± 6</td>
<td>114 ± 14</td>
<td>110 ± 0</td>
<td>98 ± 2</td>
<td>75 ± 17</td>
</tr>
<tr>
<td>Benzo[a]-pyrene</td>
<td>0.004</td>
<td>148 ± 5</td>
<td>982 ± 45</td>
<td>127 ± 7</td>
<td>832 ± 7</td>
<td>178 ± 2</td>
<td>913 ± 21</td>
<td>133 ± 11</td>
</tr>
<tr>
<td>2,4,7TNF</td>
<td>0.0006</td>
<td>536 ± 16</td>
<td>150 ± 18</td>
<td>557 ± 24</td>
<td>129 ± 8</td>
<td>506 ± 2</td>
<td>139 ± 7</td>
<td>459 ± 11</td>
</tr>
<tr>
<td>None</td>
<td>122 ± 6</td>
<td>156 ± 7</td>
<td>124 ± 2</td>
<td>127 ± 7</td>
<td>116 ± 4</td>
<td>133 ± 2</td>
<td>121 ± 3</td>
<td>136 ± 5</td>
</tr>
</tbody>
</table>

* a Standard Ames plating assay without preincubation. With and without 0.5 ml Aroclor 1254-induced rat liver S9 mix (1).

* b Same as a with preincubation for 20 min at 37°C.

* c Same as b with preincubation for 20 min at 37°C.

* d Procedure of Yahagi et al. (26, 27) with preincubation for 20 min at 37°C. With and without 0.5 ml Aroclor 1254-induced rat liver S9 mix.

* e "Complete azo dye protocol" of Prival and Mitchell (19) with and without uninduced hamster liver S9 mix with preincubation for 30 min at 30°C.

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**FIGURE 1.** Response of *S. typhimurium* TA100 to metanil yellow. Data from protocol V without S9 from Table 3.

**FIGURE 2.** Response of *S. typhimurium* TA100 to orange II. Data from protocol V without S9 from Table 3.
Strain TA100

Both dyes failed to yield mutagenicity with the standard Ames assay without preincubation and without Aroclor 1254-induced rat liver S9 mix (Table 3). Preincubation without S9 mix resulted in an approximate twofold increase in revertants over controls with either dye (Table 3, protocol II). The addition of riboflavin (21) to preincubation mixtures with and without Aroclor 1254-induced rat liver S9 failed to yield mutagenicity with either dye (Table 3, protocol III). Protocol IV without S9 resulted in lower numbers of revertants with both dyes compared to protocol II without S9 (Table 3). Protocol V with uninhibited hamster S9 resulted in the highest number of revertants observed (Table 3). The absence of hamster S9 and the presence of FMN in the preincubation mixture of protocol V increased the mutagenicity of metanil yellow 4.4-fold and that of orange II 3.8-fold respectively over that of controls (Table 3, Figures 1 and 2). Inhibition of lawn development occurred when the total quantity of both dyes in assay systems was 3 μmol, which resulted in reduced numbers of revertant colonies (Table 3).

DISCUSSION

Metanil yellow has previously been shown to be carcinogenic in mice (17). This dye has also been shown to produce dominant lethal mutations (20) and chromosomal aberrations (18). Data is not available regarding the carcinogenesis of orange II; however, this dye has been shown to elicit chromosomal aberrations (16) and dominant lethal mutations (20) in mice. The reduction product of orange II (1-aminonaphthol) has also been reported to induce bladder tumors (3, 4). Orange II has been reported to be nonmutagenic in several studies with the Ames assay (6, 12, 15) and to be negative in the prophage induction test and the Escherichia coli recA assay (12). Chung et al. (7), however, reported weak mutagenicity with S. typhimurium TA1538. There are no previous reports regarding the mutagenicity of metanil yellow with the Ames assay. Lack of mutagenicity of metanil yellow however has been reported in the yeast genotoxicity conversion assay (14) and in the E. coli recA assay (22).

Preincubation was found to be an absolute requirement for detection of mutagenicity with both dyes. The presence of Aroclor 1254-induced rat liver S9 was found to eliminate mutagenicity with both dyes as it does with orange no. 17 (15) and with benzidine-derived dyes (19). The addition of FMN to preincubation mixtures in the absence of S9 preparations was found to yield the highest numbers of revertants with both dyes. Studies with polymeric monoazo dyes have indicated that low-potential (E0 = -200 to -300 mV) electron carriers, particularly flavins, favor azo reduction (5). Evidence is available indicating in vivo azo dye reduction involving liver cytosolic and microsomal NADPH-dependent azo reductases (24). The gastrointestinal tract has also been found to reduce azo dyes in a process mediated by the intestinal microflora, probably via nonenzymatic electron-shuttle mechanisms (25). Our results involving the addition of FMN are consistent with the latter observation. The "complete azo dye protocol" involves the inclusion of FMN and the incorporation of 0.5% dextrose in the bottom agar. This is in contrast to the absence of FMN and the inclusion of 2.0% dextrose used in the standard Ames assay. Our results clearly indicate that these dyes will not be notably mutagenic unless suitable conditions are provided in the assay for their reduction. The involvement of primarily a base-pair substitution mechanism with both dyes is also evident. The experimental variables involving preincubation time, static versus agitated preincubation, dextrose concentration, and the presence and absence of FMN under these various conditions are presently being studied.

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REFERENCES