Inhibitory Effects of Food-Coloring Agents Derived from *Monascus* on the Mutagenicity of Heterocyclic Amines

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Red and yellow pigments extracted from *Monascus anka* and *Monascus purpureus* (collectively called Beni-Koji) are being used for food coloration. In the Ames *Salmonella* assay, these pigments themselves showed individually no mutagenic activity, and both of them inhibited the mutagenicity of 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole [Trp-P-2(NHOH)], the activated form of Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole). Laccaic acid and kaki pigment also inhibited the mutagenicity of Trp-P-2(NHOH). *Monascus* dyes, both yellow and red, inhibited the mutagenicity of metabolically activated 2-amino-3-methylimidazo[4,5-*f*]quinoline, 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline, and cooked-meat extract. Incubation of Trp-P-2(NHOH) with the *Monascus* dyes resulted in a rapid disappearance of the mutagen. Therefore, a role of the dyes in the inhibition of the mutagenicity appears to be acceleration of the decomposition of the activated mutagens.

Keywords: *Antimutagenicity; food colorant; heterocyclic amine; Monascus; laccaic acid; degradation of mutagen*

INTRODUCTION

Several pigments derived from natural substances are widely used in Japan as food colorants (Pharmaceutical Society of Japan, 1990). Chlorophyllin, a green colorant, is known to inhibit the mutagenicity of a variety of compounds (Ong et al., 1986; Arimoto et al., 1995; Park et al., 1995), and it suppresses the carcinogenicity of several compounds in animal models (Breinholt et al., 1995; Guo et al., 1995; Hasegawa et al., 1995) . Antimutagenic and chemopreventive effects of curcumin (a yellow pigment) are also reported (De Flora et al., 1994; Tanaka et al., 1994; Rao et al., 1995). β-Carotene (yellow) has been used in experimental, clinical, and epidemiological studies for prevention of cancer (Lippman et al., 1994; The α-Tocopherol, β-Carotene Cancer Prevention Study Group, 1994).

Recently we had an opportunity to acquire a set of individual components of several "natural" coloring agents, which included *Monascus* yellow and red pigments. Yasukawa et al. (1996) reported that oral administration of *Monascus* pigments suppressed tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in mice following initiation by 7,12-dimethylbenz[*a*]anthracene.

To explore the antimutagenicity of a food component, we have often adopted an in vitro assay system, in which the agent's capability to inhibit direct-acting mutagenicity of heterocyclic amines is tested. The carcinogenic heterocyclic amines are present in cooked meat and fish [for reviews, see Sugimura (1985) and Hayatsu (1991)] and are mutagenic to bacteria in the presence of the rat liver microsomal (S9) fraction. To avoid possible complication by the inclusion of S9 in the antimutagenicity assay, we routinely use in these assays heterocyclic amines already activated by preincubation with S9 (therefore, direct-acting). We report here the inhibitory effects of the *Monascus* pigments and other naturally occurring food colorants against the direct-acting mutagenicity of 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole [Trp-P-2(NHOH)], a primary metabolite of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), and of several heterocyclic amine-derived direct-acting mutagens, as assayed by the *Salmonella* mutagenicity test (Ames et al., 1975). Studies on the mechanism of inhibition by *Monascus* pigments are also reported.

MATERIALS AND METHODS

Monascus pigments (Sato et al., 1992) are the products from microorganisms *Monascus anka* and *Monascus purpureus* (collectively called Beni-Koji, to be used for producing Sake by fermentation). *Monascus* yellow is composed mainly of xanthomonasin A and xanthomonasin B (Figure 1). The molar ratio of xanthomonasin A and xanthomonasin B in the samples we used was 3:2. *Monascus* red is a mixture of amino acid-condensed derivatives of rubropunctatin and monascorbrin; the material we used was a mixture of glycylrubropunctatin and glycylmonascorbrin (Figure 1). Laccaic acids A−C (Figure 1) are anthraquinones (Yamada et al., 1989) produced by *Laccifer lacca* Kerr. The major pigment in red beets (*Beta vulgaris*) is betanin (Adachi and Nakatsukasa, 1983). The pigment from blueberry is a mixture of anthocyanines. The major yellow pigment from carthamine (*Carthamus tinctorius* L.) is a chalcone. The major pigment in tamarind (*Tamarindus indica* L.) is an anthocyanin, cyanidin-3-glucoside. The yellow pigment from Gardenia (*Gardnia jasminoides* Ellis) is crocin, a carotenoid. The chemical structures of gardenia blue and kaki pigment are not known. These pigments, except for laccaic acids, are the products of Yaegaki Zymotechnics (Himeji). Laccaic acids A (98% pure), B (95% pure), and C (93% pure) were gifts from Wako Pure Chemicals (Tokyo). All of these pigments are being used as food colorants in Japan. Trp-P-2, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) were purchased from Wako Pure Chemicals. Trp-P-2(NHOH) was synthesized from Trp-P-2 as described by Saito et al. (1983).

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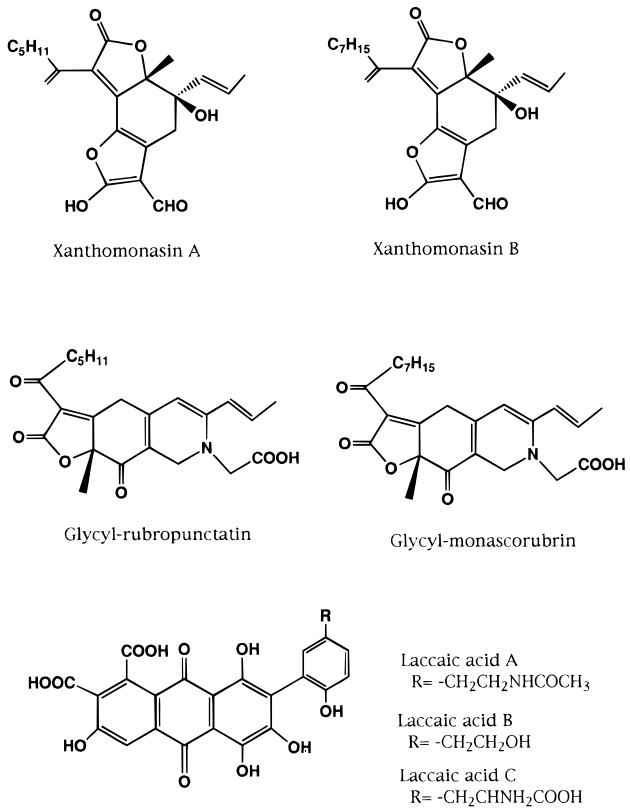

Figure 1. Structures of food-coloring agents.

Mutagen-containing extract of cooked meat was prepared as described previously (Hayatsu et al., 1983): briefly, pan-cooked ground beef was extracted with 0.1 N HCl, the extract was neutralized and treated with blue rayon to adsorb polycyclic compounds, and the blue rayon was eluted with methanol/ammonia. On evaporation of the solvent, an oily substance, which contained mutagens, was obtained. Direct-acting mutagens from IQ, MeIQ, and the cooked-meat extract (hereafter referred to as "activated IQ", "activated MeIQ", and "activated cooked-meat extract") were obtained by the method described earlier (Arimoto et al., 1980): briefly, a mutagen in 10 mM sodium phosphate buffer at pH 7.4 was treated with S9 (see below) for 20 min at 37 °C, an equal volume of cold acetone was added to the solution, the soluble fraction of the mixture was separated and evaporated under reduced pressure, and the residue obtained was dissolved in cold water. These activated mutagens were stored at −80 °C until use. *Salmonella typhimurium* TA 98 and TA 100 were gifts from Dr. B. N. Ames of the University of California, Berkeley. For enzymatic activation, polychlorinated biphenyl (PCB-54, Tokyo Kasei Chemical Co., Cl content ca. 54%)-induced rat S9 was used (Ames et al., 1975).

Mutagenicity Assays and Inhibition Experiments. The preincubation method (Yahagi et al., 1977) was employed in the *Salmonella* mutagenicity assays (Ames et al., 1975). Modifying effects of colors on the mutagenicity were examined by the procedure described earlier for chlorophyllin (Arimoto et al., 1982). Briefly, the preincubation mixture was prepared by mixing the components in the following order: 50 μL of a solution of pigment in water, 500 μL of 0.1 M sodium phosphate buffer at pH 7.4, 100 μL of an overnight culture of bacteria, and finally 50 μL of a mutagen solution. After incubation for 20 min at 37 °C, molten agar was added and the mixture was poured onto a minimal agar plate. The plates were incubated for 2 nights, and the revertant colonies that resulted were counted manually. The doses of individual mutagens used were those with which 1000 or greater numbers of revertant colonies per plate were formed. When the number per plate exceeded 2000, colonies in a certain square area were counted, and from the average counts in five such areas the total number on the plate was estimated. Mutagenic activity (percent) given in the figures is a value obtained as follows:

$$\{[(\text{His}^+ \text{ revertants in the presence of pigment}) - (\text{spontaneous revertants})]/[(\text{His}^+ \text{ revertants in the absence of pigment}) - (\text{spontaneous revertants})]\} \times 100$$

The numbers of the spontaneously formed revertants were 20−53 with TA 98 and 133-155 with TA 100. In the assays, duplicate plates were used for individual dose points. In all of these experiments, we observed that the background lawns of bacteria on the plates were not impaired.

Table 1. Inhibitory Effects of Colorants on the Mutagenicity of Trp-P-2(NHOH)[a]

colorant (0.01 mg/plate)	relative mutagenicity (%)	colorant (0.01 mg/plate)	relative mutagenicity (%)
none	100	kaki	65.0
Monascus yellow	54.2	red cabbage	87.7
Monascus red	54.8	tamarind	96.4

[a] The amount of Trp-P-2(NHOH) used was 0.01 nmol, and the number of revertants per plate found in the absence of the colorants was 2260. The colorant (0.01 mg) of beet red, blueberry color, carthamine yellow, gardenia blue, or gardenia yellow did not affect the mutagenicity of Trp-P-2(NHOH).

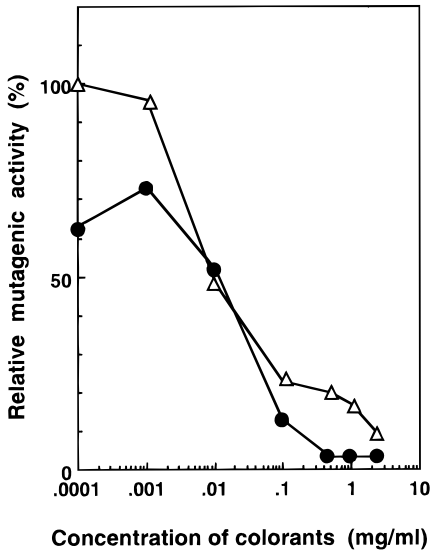

Figure 2. Effect of *Monacus* yellow (●) and *Monascus* red (△) on the mutagenicity of Trp-P-2(NHOH).

Analysis of Trp-P-2(NHOH) Decomposition. *Monascus* pigment-mediated decomposition of Trp-P-2(NHOH) was analyzed by high-performance liquid chromatography (HPLC). The reaction was started by mixing an aqueous solution of Trp-P-2(NHOH) (final concentration = 0.01 mM) with *Monascus* red (5 mg/mL) or with *Monascus* yellow (35 mg/mL) in sodium phosphate buffer (0.1 M, pH 7.4). The volume of the mixture was 0.2 mL. For a time course study, a set of five reaction tubes was used, with each tube assigned to a certain reaction time. The first tube was used for a 0-time control: immediately after the mixing, a 0.01 mL sample was taken and injected into HPLC to quantify Trp-P-2(NHOH). When the HPLC elution was near completion (about 30 min), the reaction in the second tube was started, and after 5 min of incubation at 37 °C, a 0.01 mL sample was taken and submitted to the HPLC. Reactions for 10, 20, and 30 min were analyzed similarly. The HPLC was run on a reversed phase column, Inertsil ODS (Tosoh), attached to a Waters 6000A system. The elution was done with 40% acetonitrile/20 mM sodium acetate at pH 4.5 with a flow rate of 1 mL/min. The eluent was monitored by a UV detector (Model 440) at 260 nm (Arimoto et al., 1982, 1987). The retention time of Trp-p-2(NHOH) was

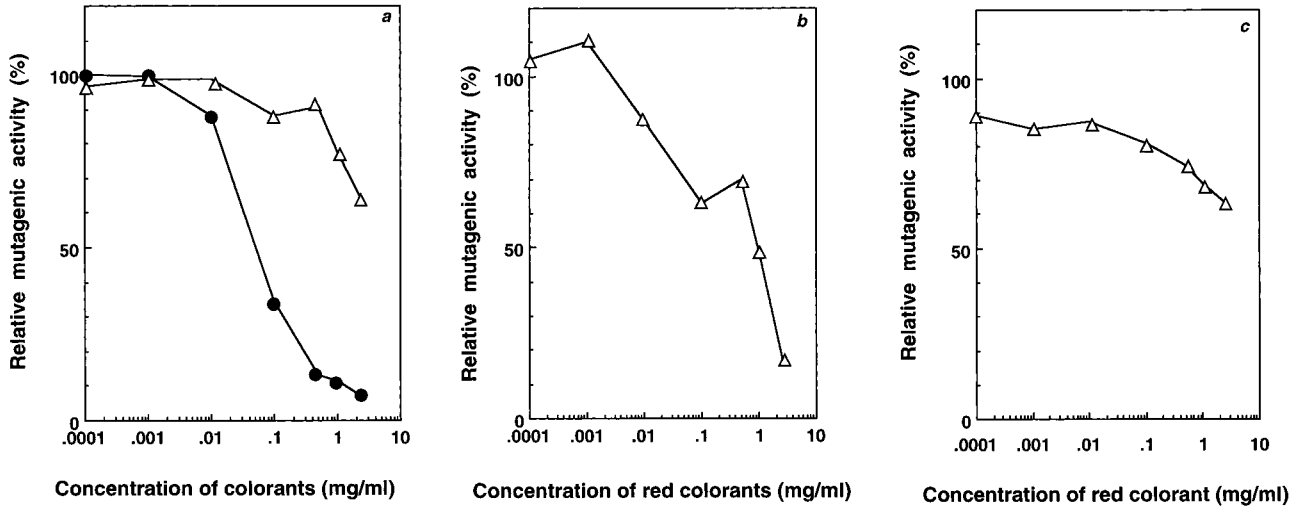

Figure 3. (a) Effect of *Monascus* yellow (●) and *Monascus* red (△) on the mutagenicity of the activated MeIQ. (b) Effect of *Monascus* red on the mutagenicity of activated cooked-meat extract. (c) Effect of *Monascus* red on the mutagenicity of the activated IQ.

Table 2. Assay of Monascus Colorants on *Salmonella typhimurium* Strains

amt of colorant, mg/plate	no. of revertants			
	TA 98		TA 100	
	+ S9	− S9	+ S9	− S9
	Yellow Colorant			
5	27	38	128	92
1	48	45	128	92
0.5	43	49	117	121
0.05	39	29	103	93
0.005	26	33	88	119
0.0005	25	19	110	198
0	25	19	110	166
	Red Colorant			
5	81	27	209	187
1	48	36	161	143
0.5	33	26	146	175
0.05	36	29	137	165
0.005	55	34	152	156
0.0005	54	39	142	176
0	58	35	131	154

3.9 min. The results reported in this paper are averages of at least twice repeated experiments.

To investigate direct, immediate interactions between Trp-P-2(NHOH) and *Monascus* yellow, the absorption spectra were measured with a Hewlett-Packard 8450A UV−vis spectrophotometer for a freshly prepared solution containing Trp-P-2(NHOH) (2.7 μM) and/or *Monascus* yellow (1.3 μg/mL, 3.5 μM) in sodium phosphate buffer (0.1 M, pH 7.4).

RESULTS AND DISCUSSION

Effects of coloring agents on the mutagenicity of Trp-P-2(NHOH) are shown in Table 1. *Monascus* yellow, *Monascus* red, and kaki pigment lowered the mutagenicity of Trp-P-2(NHOH) to 55−65%, at doses of 0.01 mg/plate. Red cabbage color seemed to suppress the activity slightly, whereas the other six coloring agents did not affect the activity at this dose. Therefore, *Monascus* colors were studied further. Both yellow and red colors showed no mutagenicity toward *S. typhimurium* TA 98 and TA 100 up to 5 mg/plate in the Ames test (Table 2). The dose−inhibition relationships for *Monascus* pigments on the mutagenicity of Trp-P-2(NHOH) are shown in Figure 2. The effects of *Monascus* pigments on the mutagenicity of several other direct-acting mutagens from food pyrolysates are given in Figure 3. *Monascus* yellow inhibited strongly the mutagenicity of activated MeIQ: 95% inhibition with 0.5 mg of the dye. *Monascus* red inhibited the mutagenicity of cooked-meat dose dependently up to 85%, and it showed a weak antimutagenicity toward the activated IQ and MeIQ (Figure 3). Xanthomonasins A and B, components of *Monascus* yellow, showed strong antimutagenic properties against Trp-P-2(NHOH), as shown in Figure 4a. Their activities were almost equivalent to each other, as might be expected from the similarity of their structures. Glycylrubropunctatin and glycylmonascorbrin, components of *Monascus* red, also showed almost the same suppressing effect against Trp-P-2(NHOH) (Figure 4b). Obviously the difference in side chains in these compounds does not affect the antimutagenic character. Laccaic acids A−C were inhibitory against Trp-P-2(NHOH), but the effects were weaker than those of the *Monascus* pigments (Figure 5).

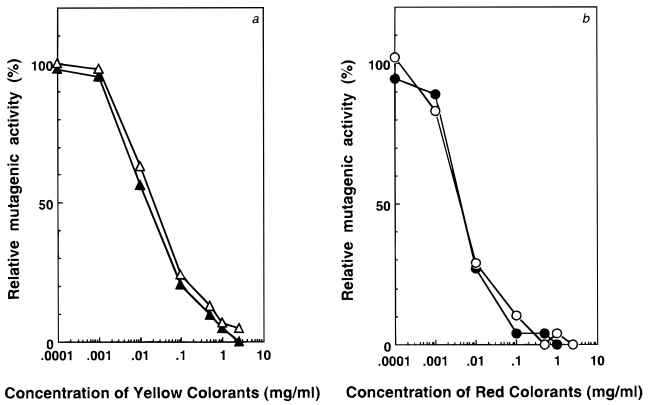

Figure 4. (a) Effect of the components of *Monascus* red pigments, xanthomonasin A (▲) and xanthomonasin B (△), on the mutagenicity of Trp-P-2 (NHOH). (b) Effect of the components of *Monascus* yellow pigments, glycylrubropunctatin (●) and glycylmonascorbin (○), on the mutagenicity of Trp-P-2(NHOH).

To elucidate the inhibition mechanisms of *Monascus* pigments, we investigated the effect of the pigments toward the stability of Trp-P-2(NHOH). It is known that Trp-P-2(NHOH) is unstable in neutral aqueous solution (Arimoto et al., 1987). A mixture of Trp-P-2(NHOH) and the pigment in a solution at pH 7 was incubated at 37 °C and analyzed by HPLC. The peak corresponding to Trp-P-2(NHOH) decreased with the time of incubation at 37 °C; no new peaks, which could include Trp-P-2, were produced (Figure 6). The amount

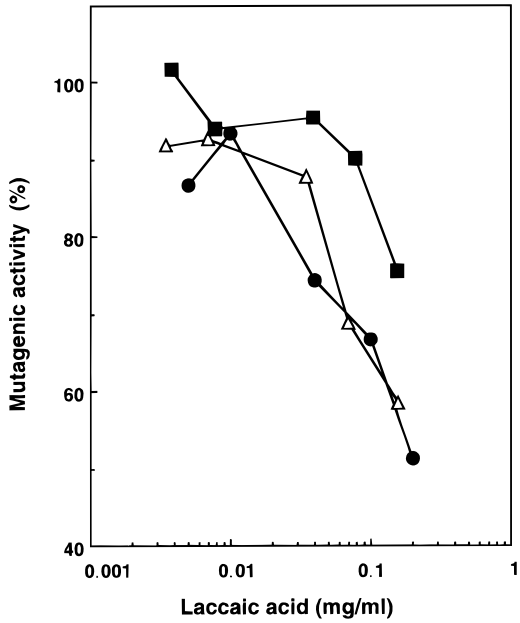

Figure 5. Effect of laccaic acids on the mutagenicity of Trp-P-2(NHOH): laccaic acid A (●); laccaic acid B (△); and laccaic acid C (■).

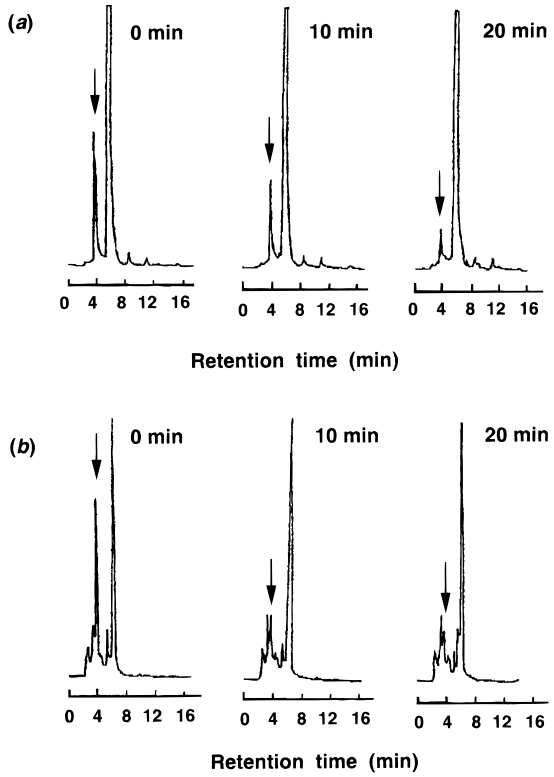

Figure 6. HPLC profiles of the degradation of Trp-P-2(NHOH) incubated with *Monascus* red (a) and *Monascus* yellow (b). The arrow indicates the peak of Trp-P-2(NHOH). The large peaks at 6.5 min are those of the pigment.

of pigment in the mixtures was unchanged during the incubation, as judged from the peak areas in the HPLC profiles. As shown in Figure 7, *Monascus* pigments accelerated the disappearance of Trp-P-2(NHOH). After 5 min of incubation with *Monascus* red, the remaining amount of Trp-P-2(NHOH) was a half of that in the spontaneous degradation. With *Monascus* yellow, there was again the acceleration but it was much smaller than with the red.

The findings described above suggest that there is molecular interaction between the colorants and Trp-P-2(NHOH). We measured the absorption spectra of Trp-P-2(NHOH), *Monascus* yellow, and a mixture of them. To avoid complication due to the degradation of Trp-P-2(NHOH), the spectra were recorded immediately after the solutions were prepared. As the spectra in Figure 8 show, significant interactions seem to be present between Trp-P-2(NHOH) and *Monascus* yellow.

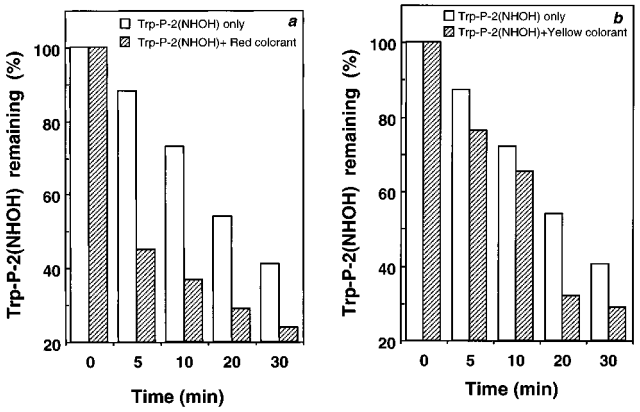

Figure 7. *Monascus* red (a) and *Monascus* yellow (b)-mediated disappearance of Trp-P-2(NHOH), as analyzed by HPLC.

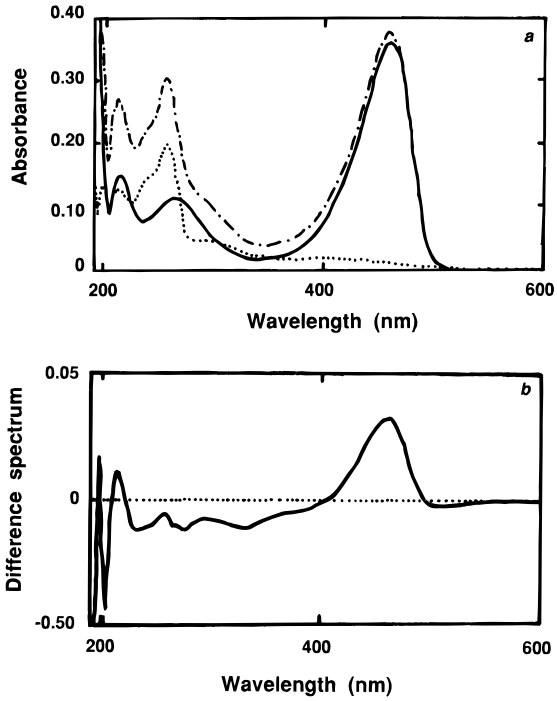

Figure 8. (a) Absorption spectra of Trp-P-2(NHOH) (···), *Monascus* yellow (—), and their mixture (— · —). (b) Difference spectrum for a mixture of Trp-P-2(NHOH) and *Monascus* yellow, derived from the curves shown in (a).

We showed previously that hemin and its related pyrrole pigments can suppress Trp-P-2 mutagenicity (Arimoto et al., 1980). Hemin and hemoglobin can accelerate the degradation of Trp-P-2(NHOH) (Arimoto et al., 1987; Arimoto and Hayatsu, 1989). It seems likely that the inhibitions observed in the present work also arise by the enhanced degradation of the ultimate active metabolites. The pigment-mediated acceleration of the degradation of Trp-P-2(NHOH) may be due to some molecular interactions, the nature of which is unknown at this stage of investigation.

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ), one of the carcinogenic cooked-meat heterocyclic amines, has

been shown to form DNA adducts in colonic mucosa of rats, due to the intake of the carcinogen from the digestive tract followed by metabolic activation inside the colonic cells (Kajikawa et al., 1995). We observed that direct-acting mutagenicity emerged in the sera of mice that had been administered intravenously Trp-P-2 and that the mutagenicity was maintained for a period of 0.5−6 h after the administration (Aji et al., 1994). Since Trp-P-2 itself is an indirect mutagen, the direct activity detected in the sera should have represented the presence of metabolically activated Trp-P-2. It is known that Trp-P-2(NHOH) is a primary metabolite of Trp-P-2 and is a direct-acting mutagen (Ishii et al., 1980). On the basis of these facts, we believe that the results reported here could be relevant in real biological settings.

The present finding that a series of food-coloring agents from natural substances can inhibit the mutagenicity of activated forms of food pyrolysate mutagens suggests that further screening of colors in plants and other natural sources for their antimutagenic activities is important.

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