

Some of the Food Color Additives Are Potent Inhibitors of Human Protein Tyrosine Phosphatases

Suja Shrestha, Bharat Raj Bhattarai, Keun-Hyeung Lee,* and Hyeongjin Cho*

Department of Chemistry and Institute of Molecular Cell Biology, Inha University, Incheon 402-751, Korea

*E-mail: khlee@inha.ac.kr; hcho@inha.ac.kr

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Synthetic color additives approved for general food use are sixteen in European Union, seven in U. S. A. and twelve in Japan. Twelve food dyes were examined for their inhibitory potency against human protein tyrosine phosphatases (PTPases). Half of the food colorants inhibited PTPases significantly and three of them were potent inhibitors with low micromolar IC₅₀ values. Also examined were the synthetic dyes structurally similar but not allowed in food. Some of them were potent inhibitors of PTPases. Considering the importance of PTPases in cellular signal transduction, inhibition of PTPases by food colorants might cause harmful effects in human health.

Key Words : Food color additive, Certified color, Dye, Protein tyrosine phosphatase, Enzyme inhibitor

Introduction

Color additives are used in a wide variety of foods such as beverages, dairy products, cereals, bakery goods, snack foods and ice creams. The use of the additives is strictly controlled in most of the countries by food sanitation laws. Colors permitted for use in foods are generally classified as certified or exempt from certification.¹ Certified colors are man-made and there are seven certified colors approved for general food use in the United States and twelve colors approved in Japan (Table 1). In European Union (EU), sixteen synthetic colors are approved and eight of them are listed in Table 1. Color additives that are exempt from certification include pigments derived from natural sources such as vegetables, minerals or animals.¹

Although food colors are carefully regulated to ensure their safety, not all colors are harmless and their adverse side effects have been reported. For example, anaphylaxis, hyper-

tension, hypotension and teratogenicity were reported for Indigocarmine.²⁻⁵ Anaphylaxis due to carmine allergy was also reported.⁶ Rose Bengal is known to affect IgE production.⁷ This dye and other artificial food colors may be cytotoxic to hepatocytes.^{8,9} Fast Green FCF inhibits synaptic activity in rat hippocampal interneurons and Tartrazine stimulates mitogenic process.^{10,11} Azo dyes such as Amaranth, Allura Red and New Coccine induced colon DNA damage in rodents.¹² A few food colors in combination with other additives produced adverse effects in platelet function.¹³ The relevance of food additives in the pathogenesis of atopic dermatitis was also suggested.¹⁴ The mechanism of these observations is largely unknown in most of the cases.

While studying the enzymology of protein tyrosine phosphatases (PTPases), we observed that some of the dyes behaved as potent inhibitors of PTPases.^{15,16} PTPases are a class of enzymes that hydrolyze the phosphate moiety from a phosphotyrosine residue of a protein thus regulating the

Table 1. Synthetic food colorants permitted by the Food Sanitation Laws of U. S. A. (1-7)^a, Japan (1-3, 4-12) and European Union (1-2, 4-9)

Comps.	FDA name ^b	Japanese name	E No. ^c	Common name	CAS No.	CI No. ^d
1	FD&C Blue No. 1	Food Blue No. 1	E-133	Brilliant blue FCF	3844-45-9	42090
2	FD&C Blue No. 2	Food Blue No. 2	E-132	Indigotine	860-22-0	73015
3	FD&C Green No. 3	Food Green No. 3		Fast green FCF	2353-45-9	42053
4	FD&C Red No. 3	Food Red No. 3	E-127	Erythrosine	16423-68-0	45430
5	FD&C Red No. 40	Food Red No. 40	E-129	Allura Red AC	25956-17-6	16035
6	FD&C Yellow No. 5	Food Yellow No. 4	E-102	Tartrazine	1934-21-0	19140
7	FD&C Yellow No. 6	Food Yellow No. 5	E-110	Sunset Yellow FCF	2783-94-0	15985
8	D&C Red No. 2	Food Red No. 2	E-123	Amaranth	915-67-3	16185
9		Food Red No. 102	E-124	New Coccin	2611-82-7	16255
10	D&C Red No. 28	Food Red No. 104		Phloxine B	18472-87-2	45410
11		Food Red No. 105		Rose Bengal	632-69-9	45440
12		Food Red No. 106		Acid Red 52	3520-42-1	45100

^aAmong the nine certified colors approved for food use in U. S. A., Orange B and Citrus Red No.2 are restricted to specific uses and they are not listed in this table. ^bIn FD&C and D&C, capital letters represent food, drug and cosmetics, respectively. ^cIn European Union, sixteen synthetic colors are approved and seven of them are listed in this Table. ^dCI No. abbreviates color index number.

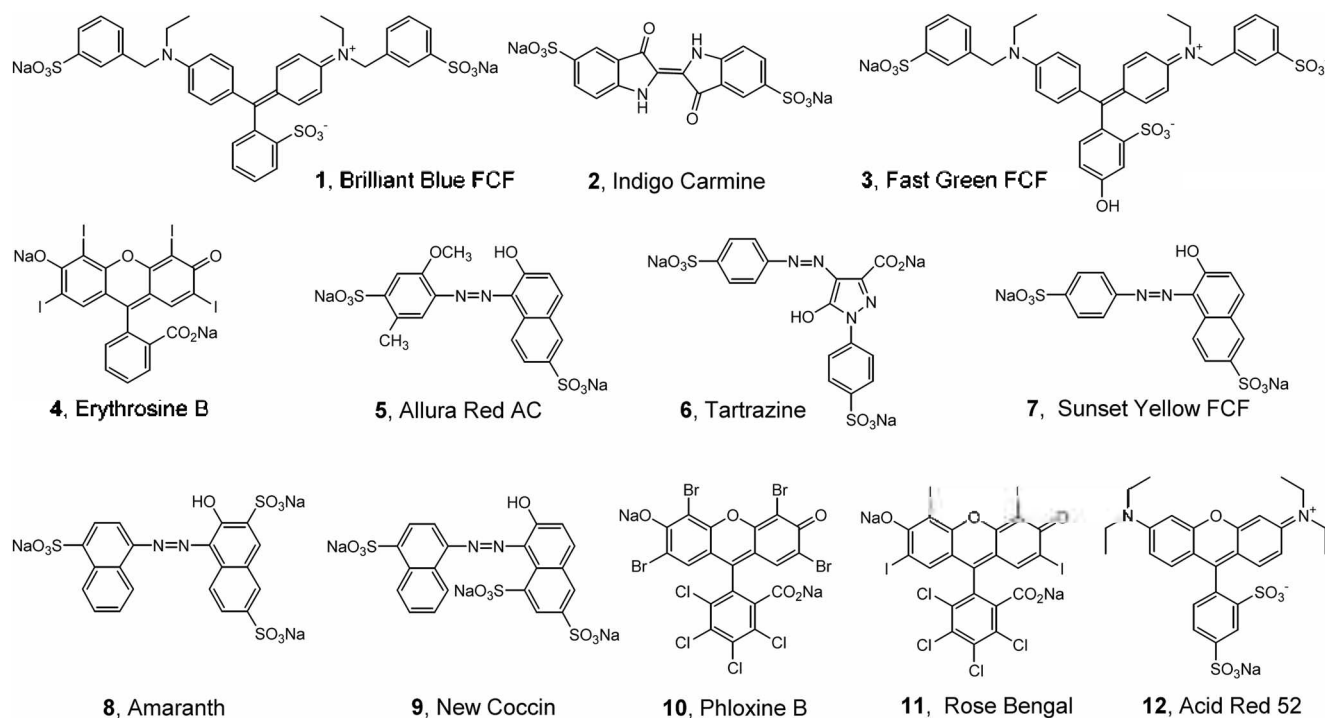


Figure 1. Chemical structure of synthetic food colorants permitted by the Food Sanitation Laws of U. S. A., European Union and/or Japan.

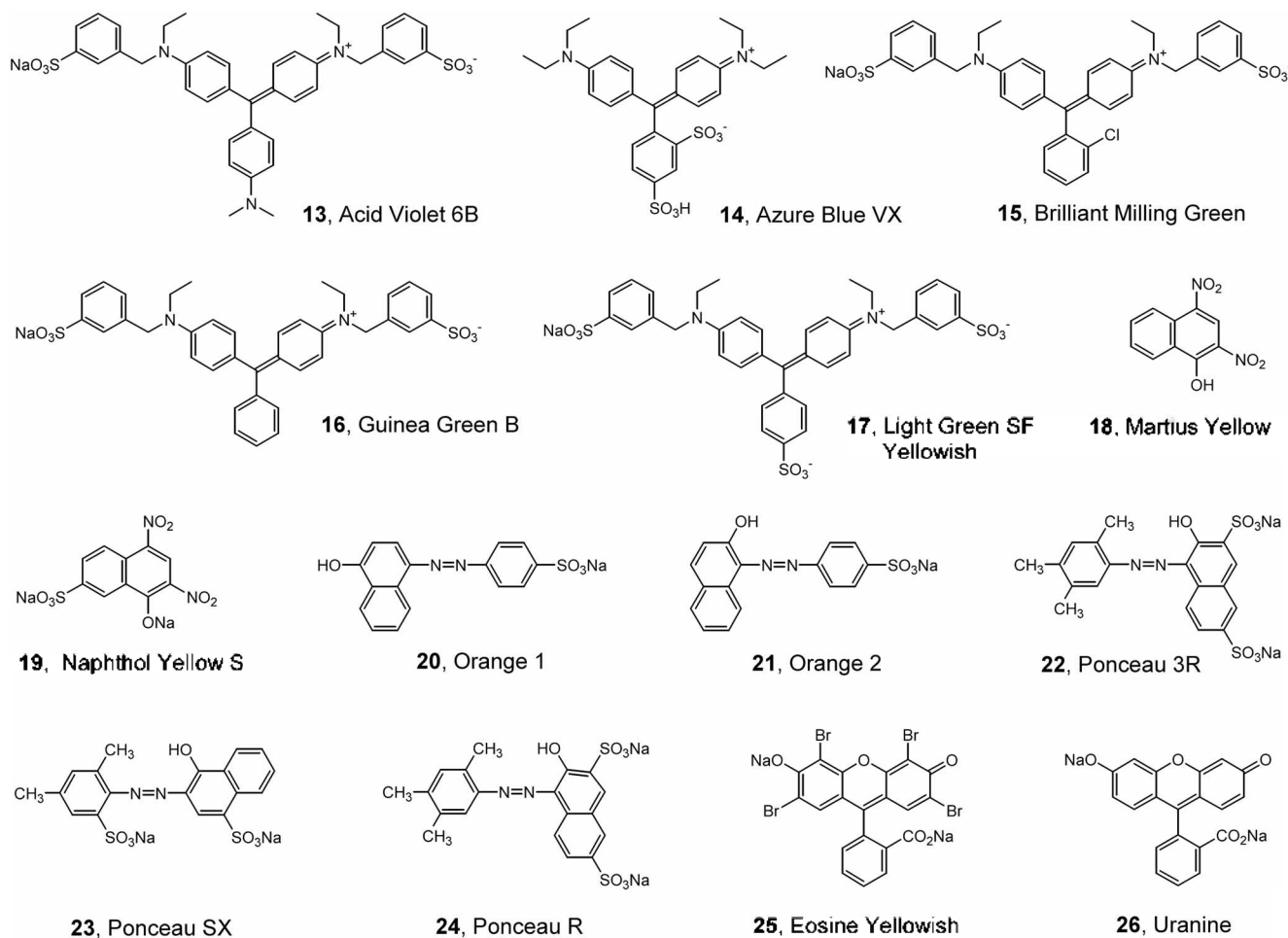


Figure 2. Chemical structure of food-forbidden colorants tested in this study.

cellular phosphorylation level of proteins in collaboration with protein tyrosine kinases.^{17,18} Because PTPases play important roles as regulators of a diverse of signal transduction pathways, inhibition of PTPases by food colorants might disturb a certain signaling pathways resulting in adverse effects in human health. Therefore, we evaluated the inhibitory potency of the certified food colorants against PTPases (1-12, Table 1, Figure 1). Also examined were the dyes not allowed in food but they are structurally similar with the certified food colorants (13-26, Figure 2).

Results and Discussion

Concentrations of the colorants for half-maximal inhibition (IC_{50}) of the *p*-nitrophenyl phosphate (*p*NPP) phosphohydrolase activity were measured against two human PTPases, PTP1B and TC-PTP, and a microbial PTPase YPTP1.^{15,19} As shown in Table 2, broad spectrum of IC_{50} values was observed both in food-permitted (1-12) and food-forbidden colorants (13-26). Although the colorants 13-26 are not

Table 2. Inhibition of PTPases by various synthetic dyes. Compounds 1-12 are permitted to add in foods in U. S. A. (1-7), Japan (1-3, 4-12) and/or E. U. (1, 2, 4-9). Compounds 13-26 are prohibited to be used in food

Compounds	IC_{50} (μM) ^a		
	PTP1B	TC-PTP	YPTP1
1	91 ± 23	>120	>120
2	110 ± 8	85 ± 11	130 ± 28
3	>124	>120	>120
4	6.0 ± 0.6	24 ± 1	13 ± 2
5	33 ± 6	67 ± 1	410 ± 80
6	>190	>190	>190
7	>220	>220	>220
8	34 ± 7	43 ± 2	103 ± 15
9	>170	>170	>170
10	4.2 ± 0.3	14 ± 1	5.2 ± 0.7
11	3.0 ± 0.3	4.0 ± 0.2	3.0 ± 0.2
12	>170	>170	>170
13	8.0 ± 0.6	46 ± 2	16 ± 2
14	>180	>180	>180
15	5.0 ± 0.4	27 ± 4	10 ± 1
16	2.2 ± 0.2	9.0 ± 0.3	7.5 ± 0.4
17	4.0 ± 0.3	9.0 ± 0.3	11 ± 1
18	242 ± 74	>430	>430
19	>280	>280	>280
20	47 ± 4.5	87 ± 5	76 ± 7
21	134 ± 24	>290	>290
22	60 ± 8.3	115 ± 6	69 ± 13
23	>210	>210	>210
24	139 ± 14	>210	68 ± 6
25	63 ± 6.8	>140	>140
26	>270	>270	>270

^a IC_{50} values were usually derived from duplicates or more of experiments using a range of inhibitor concentrations. The numbers indicate mean value ± standard deviation

allowed to be used in foods, most of them have FD&C or D&C numbers indicating that they had previously been used in foods or they are currently used in drugs and/or cosmetic products. The dyes in this study could be classified arbitrarily into three groups - potent inhibitors ($IC_{50} < 10 \mu M$), medium potency inhibitors ($IC_{50} = 30-70 \mu M$) and poor inhibitors ($IC_{50} > 100 \mu M$). Diverse of colorants in this study can also be grouped according to the structural features; triphenylmethane dyes (1, 3, 13-17), azo dyes (5, 6-9, 20-24), xanthene dye (4, 10-12, 25, 26) and others (2, 18, 19) (Table 3). Food-permitted and food-forbidden colorants are evenly distributed in these structural groups. It is worth to note that the food dyes Erythrosine (4), Phloxine (10) and Rose Bengal (11) were potent inhibitors with IC_{50} values of 6.0 μM , 4.2 μM and 3.0 μM respectively against PTP1B. Rose Bengal, the most potent among the three dyes, also strongly inhibited TC-PTP and YPTP1 with IC_{50} values of 4.0 μM and 3.0 μM respectively. The potent food inhibitors 4, 10 and 11 all belong to xanthene dyes compared to the potent food-forbidden dyes all of which belong to triphenylmethane dyes (Table 3).

As for the selectivity in the inhibition of the enzymes, the colorants exhibited less than four-fold differences in the IC_{50} values against the three PTPases. Exceptions were Allura Red AC (5) which exhibited 12-fold PTP1B selectivity against YPTP1 and Brilliant Milling Green (15) which exhibited 5.4-fold PTP1B selectivity against TC-PTP. The absence of discrimination between the PTPases might be understood on the basis of the structural similarity between the PTPases.²⁰ Selective inhibition of a PTPase among dozens of PTPases present in human is a challenging hurdle in the development of a novel therapeutic agent targeting a certain PTPase - e.g. PTP1B inhibitor development for the treatment of type II diabetes.²¹

To investigate the mode of inhibition by the dyes, steady-state kinetic experiments of PTP1B and YPTP1 were performed for Indigo Carmine, Amaranth, Rose Bengal and Guinea green B. They were the dyes with the lowest IC_{50} in each of the structural groups as classified in Table 3. The mode of inhibition was determined by the Lineweaver-Burk plot analysis of the results of the kinetic experiments (Figure 3). As shown in Table 4, Indigo Carmine and Amaranth inhibited both PTP1B and YPTP1 competitively indicating that they bind to the active site of the enzymes. Rose Bengal and Guinea green B were mixed type inhibitors of PTP1B

Table 3. Classification of synthetic dyes according to the chemical structure and the potency of PTPase Inhibition. Food colorants (1-12) are shown in italic

Structural groups	Potent ^a	Medium ^a	Poor ^a
Triphenylmethane dye	<i>13, 15-17</i>		<i>1, 3, 14</i>
Azo dye		<i>5, 8, 20, 22</i>	<i>6, 7, 9, 21, 23, 24</i>
Xanthene dye	<i>4, 10, 11</i>	<i>25</i>	<i>12, 26</i>
Others			<i>2, 18, 19</i>

^aPotency of PTPase inhibition was arbitrarily classified as potent ($IC_{50} < 10 \mu M$), medium ($IC_{50} = 30-70 \mu M$) and poor ($IC_{50} > 100 \mu M$).

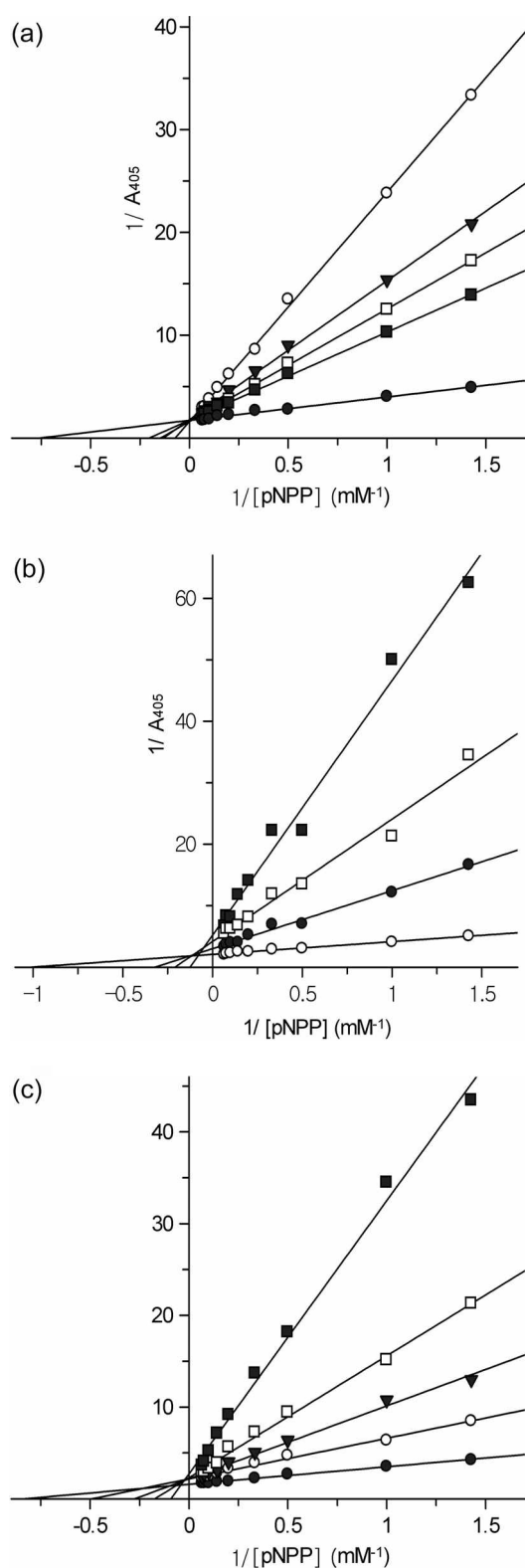


Figure 3. Lineweaver-Burk plot analysis. Phosphatase activity was measured against *p*NPP in the presence of colorants. (a) PTP1B catalyzed reactions in the presence of 100 μM (\circ), 60 μM (\blacktriangledown), 40 μM (\square), 30 μM (\blacksquare) or none (\bullet) of Amaranth (**8**). (b) PTP1B catalyzed reactions in the presence of 20 μM (\blacksquare), 15 μM (\square), 7 μM (\bullet) or none (\circ) of Rose Bengal (**11**). (c) YPTP1 catalyzed reactions in the presence of 12 μM (\blacksquare), 10 μM (\square), 8 μM (\blacktriangledown), 6 μM (\circ) or none (\bullet) of Guinea Green B (**16**).

Table 4. Nature of PTPase Inhibition by the dyes representative of the structural groups

Compounds	Dyes ^a	Nature of Inhibition	
		PTP1B	YPTP1
2	Indigo Carmine	Competitive	Competitive
8	Amaranth	Competitive	Competitive
11	Rose Bengal	Mixed	Competitive
16	Guinea green B	Mixed	Competitive

^aThe dyes selected for the kinetic experiments were those with the lowest IC_{50} value in each of the structural groups as classified in Table 3.

but inhibited YPTP1 competitively. The fact that Rose Bengal and Guinea green B compete with the substrate for the binding on YPTP1 but not on PTP1B may reflect the possible interaction of the dyes with PTP1B on the second phosphate-binding site near the active site of PTP1B.²² The second phosphate-binding site has been previously identified on PTP1B as a result of X-ray crystallographic study but the site was not present on YPTP1. Further structural study would explain the kinetic behavior of the dyes against the PTPases.

Although there are strict guidelines for chemicals to be approved as food additives, the safety of food colorants has not been rigorously proven and acceptable daily intake (ADI) has been used to minimize any possible unfavorable effect of the dyes.¹ It is interesting to note that some of the food-forbidden dyes (for example, **13**, **16**, **17**, **22**, **23**) in Figure 2 had previously been permitted for use in foods²³ and they have FD&C numbers. This fact might be extrapolated to predict that some of the currently food-permitted colorants could be forbidden sometime in the future when an unexpected toxic effect will be recognized for the dye. Considering the importance of PTPases in a diverse of cellular regulation, inhibition of those by some of the food colorants as observed in this study might initiate critical processes leading to adverse consequences on human health.

In summary, we investigated the inhibition of PTPases by certified food colorants as well as food-forbidden dyes. They inhibited the enzymes with a range of potencies. Noteworthy is that three of the food colorants inhibited the PTPases potentially suggesting the possibility of harmful effects of the certified food colorants on human health.

Experimental Section

Reagents and Enzymes. All the dyes were purchased from TCI (Tokyo, Japan) as kits of food color testing solutions A (Catalog no. F 0118) and B (Catalog no. F 0119). The test solutions A included dyes permitted in Japan for use in foods (**1-12**, Fig. 1). The test solutions B included food-forbidden dyes (**13-26**, Fig. 2). Although the latter are currently not allowed in foods, most of them have FD&C or D&C numbers. The dyes were used as received without further purification. PTP1B and YPTP1 were expressed in *E. coli* expression systems and purified as described.¹⁵⁻¹⁹ TC-PTP was purchased from New England Biolabs (Beverly,

USA). The enzymes were diluted before use to an appropriate concentration by enzyme dilution buffer (25 mM Hepes, 5 mM EDTA, 1 mM DTT, 1 mg/mL BSA, pH 7.3).

Enzyme Assay. For inhibition assay, inhibitor (5 μ L in H₂O) was added to a mixture containing enzyme (5 μ L), 5x reaction buffer (10 μ L, 0.5 M Hepes, 25 mM EDTA, 50 mM DTT, pH 7.0) and water (25 μ L) and it was incubated at 37 °C for 10 min. The reaction was initiated by addition of *p*-nitrophenyl phosphate (*p*NPP) (5 μ L, 20 mM) and, after 3 min at 37 °C, the reaction was quenched by addition of NaOH solution (950 μ L, 0.5 M). The progress of the reaction was determined for the formation of *p*-nitrophenolate by measuring the absorbance at 405 nm. The quantity of enzymes used for typical 50 μ L reaction was 200 ng for PTP1B, 30 ng for YPTP1 and 1.25 units (manufacturer's definition) for TC-PTP. The kinetic data were analyzed using GraFit 5.0 program.

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