Enhancing Effect of Carbohydrate Pyrolysates on Mutagenesis in *Salmonella typhimurium*

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Carbohydrate pyrolysates obtained at 700°C intensively enhanced mutagenicities of several mutagenic agents on *Salmonella typhimurium*. The pyrolysates obtained by heating from 1 to 15 min had an enhancing effect on the mutagenicity of a similar level. Of the 8 kinds of carbohydrates tested, sucrose pyrolysate showed the highest enhancing effect. The enhancement of mutagenicity by sucrose pyrolysate was much more effective on aromatic amines such as 2-aminofluorene and 2-aminoanthracene than on polycyclic aromatic hydrocarbons. The enhancing effect of sucrose pyrolysate was exhibited in the presence of a metabolic activation system.

Pyrolysates of protein and amino acids such as tryptophan and lysine have been reported to exhibit high mutagenicity against *Salmonella typhimurium* TA98.^{1~3)} Mizusaki *et al.*⁴⁾ have recently reported that mutagenicity of cigarette smoke condensate was due to protein and amino acids in tobacco shred during the smoking process and the mutagenic potency is dependent on the nitrogen and soluble-sugar contents of tobacco leaves. This prompted us to examine in detail interaction of pyrolysates of amino acids and sugars in exhibiting mutagenicity. The following interesting phenomena were observed in the course of this study. The pyrolytic product of a mixture of tryptophan and sucrose markedly decreased in mutagenicity on *S. typhimurium* TA98. On the contrary, when tryptophan pyrolysate was assayed simultaneously together with sucrose pyrolysate, the mutagenicity increased slightly. This paper describes the results concerning the enhancing effect of the sucrose pyrolysate on the mutagenicities

of AAF, Trp-P and other chemicals.

MATERIALS AND METHODS

Chemicals. AAF, AF, AA, DAB, PAHs, benzo(f)-quinoline, benzo(h)quinoline, 2-nitrofluorene, 4-NQO, MNNG, α -nitronaphthalene and several carbohydrates were of the purest grade available from commercial sources. The Trp-P preparation was isolated from tryptophan pyrolysate⁵⁾ and consisted essentially of 3-amino-1,4-dimethyl-5H-pyrido-(4,3-b)-indole and 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole.⁶⁾ α -Amination of quinoline, isoquinoline, benzo(f)quinoline, benzo(h)quinoline and phenanthridine was carried out with sodium amide or potassium amide.⁷⁾ *N*-hydroxy-AAF⁸⁾ and *N*-acetoxy-AAF⁹⁾ were prepared by the methods cited in the references.

Method of pyrolysis. About 2.5 mg of the sample was put into a small pyrex or quartz tube (4 mm O.D. and 0.5 mm thick), which was evacuated to about 10^{-3} mm Hg and sealed in a flame of commercial urban gas and oxygen. A pyrex tube was used in experiments lower than 600°C and a quartz tube was used in those higher than 700°C. Pyrolyses were performed in a Packard Tri-carb Combustion Furnace, Model 325. The sealed tube was inserted into the middle of the pyrolysis column maintained at designated temperatures from 100 to 800°C and kept there for 1 to 15 min. The tube after removal and cooling at room temperature was broken and 0.2 ml of DMSO was added to the contents per 1 mg of carbohydrate pyrolyzed. The DMSO solution was used for mutagenesis assay within 2 hr after pyrolysis.

Abbreviations: DMSO, dimethylsulfoxide; AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; AA, 2-aminoanthracene; BP, benzo(a)pyrene; DAB, dimethylaminoazobenzene; 4-NQO, 4-nitroquinoline-*N*-oxide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PAH, polycyclic aromatic hydrocarbon; Trp-P, mutagenic fraction from tryptophan pyrolysate.

Mutagenesis assay. *Salmonella typhimurium* test strains, TA98 and TA100, were kindly provided by Dr. B. N. Ames, University of California, Berkeley. The mutagenic testing was carried out as described by Ames *et al.*¹⁰ To a test-tube containing 2 ml of molten top agar (0.6% agar, 0.6% NaCl, 0.05 mM histidine, 0.05 mM biotin) were added 0.1 ml of the DMSO solution of the pyrolysate, a proper amount of chemical solution (0.1 ml maximum), 0.1 ml of an overnight nutrient broth culture of the bacterial test strain and 0.5 ml of a solution that contained 0.05 ml of rat liver microsomal fractions (S-9), 4 μ mol MgCl₂, 33 μ mol KCl, 2.5 μ mol glucose-6-phosphate 2 μ mol, NADPH and 50 μ mol sodium phosphate buffer (pH 7.4). The tube contents were mixed and poured onto a plate containing 1.5% agar, 2% glucose and minimal salts. Histidine-revertant colonies were counted after incubation at 37°C for 48 hr. S-9 was obtained from rats induced with polychlorinated biphenyl as described by Ames *et al.*¹⁰

RESULTS AND DISCUSSION

The effect of sucrose pyrolysate on the mutagenicity of tryptophan pyrolysate on *S. typhimurium* TA98 is shown in Fig. 1. When tryptophan was pyrolyzed in the presence of sucrose, the mutagenicity of the pyrolytic product of the mixture decreased markedly with increasing amounts of sucrose in the

FIG. 1. Effect of Sucrose Pyrolysate on the Mutagenicity of Tryptophan Pyrolysate.

The pyrolyses were performed at 500°C. Pyrolysates of a mixture of tryptophan and sucrose (○—○), and pyrolysates which were mixed after pyrolysis of tryptophan and sucrose separately (●—●) were tested for the mutagenicity using *S. typhimurium* TA98 in the presence of S-9 mix. The number of His⁺ revertants per plate was plotted against amount of sucrose pyrolyzed. To each assay medium was added the pyrolysate from 50 μ g of tryptophan.

pyrolysis. On the other hand, when tryptophan pyrolysate was assayed simultaneously

TABLE I. ENHANCING EFFECT OF CARBOHYDRATE PYROLYSATES ON THE MUTAGENICITY OF AAF TOWARD *S. typhimurium* TA98

Pyrolysate of 500 μ g carbohydrate, and 5 μ g of AAF was added to the assay mixture with S-9 mix. The number of His⁺ revertants of the each pyrolysate obtained at 700°C for 1 min was 70 on average. Non-pyrolyzed carbohydrates did not show any enhancing effect on the mutagenicity. Spontaneous revertants (55) were subtracted.

Sample	Treatment with pyrolysate of carbohydrate	Number of revertants per plate
AAF	None	259
AAF	Glucose	1304
AAF	Fructose	1427
AAF	Mannose	1237
AAF	Ribose	1388
AAF	Sucrose	1645
AAF	Maltose	1437
AAF	Cellulose	1359
AAF	Sorbitol	1122

FIG. 2. Effect of Pyrolytic Temperature and Time on the Enhancement of Mutagenicity by Sucrose Pyrolysate.

Pyrolysate of 500 μ g sucrose with (—) or without (----) addition of 3 μ g AAF per plate was tested for mutagenicity using *S. typhimurium* TA98 in the presence of S-9 mix.

together with sucrose pyrolysate, the mutagenicity of the mixture increased slightly. Addition of sucrose pyrolysate to mutagenic agents such as AAF and Trp-P also resulted in remarkable increases in the mutagenicities. In preliminary experiments, 8 kinds of carbohydrates including mono-, di- and polysaccharides were tested to determine their enhancing effects on the mutagenicity of AAF. As shown in Table I, all carbohydrates tested were found to enhance the mutagenicity and of them sucrose pyrolysate showed the highest enhancing effect. In the next experiment, sucrose was pyrolyzed at 8 different temperatures from 100 to 800°C for 1 to 15 min. As shown in Fig. 2, the enhancing effect of sucrose pyrolysate on the mutagenicity of AAF was maximum when sucrose was pyrolyzed at 600 or 700°C and the highest activity changed little with pyrolytic time from 1 to 15 min,

FIG. 3. Effect of the Doses of Sucrose Pyrolysate on the Enhancement of Mutagenicity.

Sucrose pyrolysates were tested for mutagenicity with S-9 mix in the presence of 2 μ g AF (●—●), or 0.1 μ g Trp-P (○—○), or no addition (Δ — Δ). Mutagenesis was assayed using TA98.

FIG. 4. Effect of the Dose of Chemicals on the Mutagenicity of Sucrose Pyrolysate.

Mutagenicities were tested using TA 98 and TA 100 with or without the pyrolysate corresponding to 500 μ g sucrose in the presence of S-9 mix. Mutagenicities of (a) AF, (b) AAF, (c) AA, (d) Trp-P and (e) DAB; with sucrose pyrolysate on TA 98 (●—●), without sucrose pyrolysate on TA 98 (○—○), with sucrose pyrolysate on TA 100 (●---●), without sucrose pyrolysate on TA 100 (○---○).

TABLE II. EFFECT OF SUCROSE PYROLYSATE ON THE MUTAGENICITIES OF POLYCYCLIC AROMATIC HYDROCARBONS

The mutagenicities of PAHs were tested using *S. typhimurium* TA 98 and TA 100 with or without the pyrolysate corresponding to 500 μg sucrose in the presence of S-9 mix. The number of spontaneous revertants (TA 98, 45; TA 100, 166) was subtracted.

Compound	TA 98			TA 100		
	Dose ($\mu\text{g}/\text{plate}$)	Revertants per plate		Dose ($\mu\text{g}/\text{plate}$)	Revertants per plate	
		without SP ^a	with SP ^a (500 $\mu\text{g}/\text{plate}$)		without SP ^a	with SP ^a (500 $\mu\text{g}/\text{plate}$)
Benzo(a)pyrene	2	302	543	3	392	885
3-Methylcholanthrene	10	372	498	5	614	871
Anthracene	100	16	92	100	88	228
Pyrene	500	31	54	500	64	136
Chrysene	10	47	99	5	310	720
Benz(a)anthracene	20	119	191	5	148	354
Fluorene	500	N.D. ^b	15	500	N.D. ^b	66
Phenanthrene	500	N.D. ^b	5	500	11	18
None	—	—	82	—	—	140

^a SP, sucrose pyrolysate; ^b N.D., not detected up to 500 μg .

TABLE III. EFFECT OF SUCROSE PYROLYSATE ON THE MUTAGENICITIES OF QUINOLINE DERIVATIVES

The mutagenesis assay was performed using *S. typhimurium* TA 98 and TA 100 with or without the pyrolysate corresponding to 500 μg sucrose in the presence of S-9 mix. The number of spontaneous revertants (TA 98, 38; TA 100, 154) was subtracted.

Compound	TA 98			TA 100		
	Dose ($\mu\text{g}/\text{plate}$)	Revertants per plate		Dose ($\mu\text{g}/\text{plate}$)	Revertants per plate	
		without SP ^a	with SP ^a (500 $\mu\text{g}/\text{plate}$)		without SP ^a	with SP ^a (500 $\mu\text{g}/\text{plate}$)
Quinoline	100	98	87	50	566	497
2-Aminoquinoline	100	69	155	50	322	365
1-Aminoisoquinoline	200	16	65	200	74	236
Benzo(f)quinoline	20	52	67	20	240	394
2-Aminobenzo(f)quinoline	1	241	1896	1	148	894
Benzo(h)quinoline	100	N.D. ^b	60	20	165	361
2-Aminobenzo(h)quinoline	20	58	114	1	149	445
9-Aminophenanthridine	20	100	162	20	289	461
None	—	—	69	—	—	105

^a SP, sucrose pyrolysate; ^b N.D., not detected up to 500 μg .

whereas the pyrolysates of sucrose alone were very weakly mutagenic.

Fig. 3 shows the dose-response relationship of sucrose pyrolysate on the enhancing effect on the mutagenicities of AF and Trp-P toward TA98. The number of *His*⁺ revertants induced by AF and Trp-P increased almost linearly with the dose level of sucrose pyrolysates added when they were assayed in the presence of S-9 mix. The pyrolysate cor-

responding to 500 μg sucrose per tube enhanced the mutagenicities of AF and Trp-P several times. The enhancing effect of sucrose pyrolysate on the mutagenicities of several mutagenic agents on *S. typhimurium* TA98 and TA100 is shown in Fig. 4. The pyrolysate corresponding to 500 μg sucrose was added to a tube of the assay mixture. The mutagenicities of AAF, AF, AA and Trp-P were markedly enhanced, but that of DAB was not

so much. The numbers of *His*⁺ revertants induced by AAF and Trp-P were increased 10- and 8-fold in the presence of sucrose pyrolysate, respectively. Table II shows the effect of sucrose pyrolysate on the mutagenicities of PAHs on *S. typhimurium* TA98 and TA100. The enhancing effect of sucrose pyrolysate was evident in BP, 3-methylcholanthrene, chrysene and benz(a)anthracene on

TABLE IV. EFFECT OF SUCROSE PYROLYSATE ON THE MUTAGENICITY IN THE ABSENCE OF S-9 MIX

The mutagenicities were assayed with *S. typhimurium* TA98 and TA100 in the presence of the pyrolysate corresponding to 500 μ g sucrose. The number of spontaneous revertants (TA98, 71; TA100, 200) was subtracted.

Compound	Dose ($\mu\text{g}/\text{plate}$)	Revertants per plate	
		without SP ^a	with SP ^a (500 $\mu\text{g}/\text{plate}$)
i) TA98			
2-Nitrofluorene	1	251	219
<i>N</i> -Hydroxy-AF	0.5	3162	3214
<i>N</i> -Acetoxy-AAF	5	100	114
None	—	—	63
ii) TA100			
4-NQO	1	3945	3829
MNNG	2	1850	1864
α -Nitronaphthalene	50	1138	1186
None	—	—	109

^a SP, sucrose pyrolysate.

FIG. 5. Effect of Various Amounts of S-9 on the Mutagenicity of Sucrose Pyrolysate with AAF.

The mutagenesis test was performed using TA98 and pyrolysate of 500 μ g sucrose with 3 μ g AAF (●—●) or 3 μ g AAF (○—○) or pyrolysate of 500 μ g sucrose (Δ—Δ).

TA100 and was not evident in anthracene, pyrene, fluorene and phenanthrene on both strains, but the effect on these compounds was much less than that on aromatic amines. The effect of sucrose pyrolysate on the mutagenicities of quinoline derivatives on TA98 and TA100 is shown in Table III. Of these compounds the most obvious enhancement of the mutagenicity was observed with 2-amino-benzo(f)quinoline.

The effect of sucrose pyrolysate on the mutagenicities of direct mutagens is shown in Table IV. Without S-9 mix, addition of sucrose pyrolysate did not show any enhancing effect on the mutagenicities of 2-nitrofluorene, *N*-hydroxy-AAF and *N*-acetoxy-AAF against TA98 and on those of 4-NQO, MNNG and α -nitronaphthalene against TA100. This clearly indicates that the enhancing effect of sucrose pyrolysate is exhibited in the presence of a metabolic activation system.

Next we examined the effect of different amounts of S-9 added on the enhancing action of sucrose pyrolysate with AAF on TA98. As shown in Fig. 5, the enhancing activity of sucrose pyrolysate with AAF increased up to 50 μ l of S-9, and further increases in the amount of S-9 resulted in decreases in the number of revertants. However, without sucrose pyrolysate, the number of revertants of AAF was maximum at an amount of 20 μ l S-9, and with over 20 μ l the mutagenicity of AAF decreased. On the other hand, an increase in the amount of S-9 hardly affected the mutagenicity of sucrose pyrolysate.

Sucrose pyrolysate is very weakly mutagenic. However, in the case of aromatic amines the mutagenicity is much higher than expected from the sum of revertants induced by the mutagenic agents and those by sucrose pyrolysate. Matsumoto *et al.*¹¹⁾ and Nagao *et al.*^{12~14)} recently reported that harman and norharman, which were not mutagenic, showed comutagenic actions to Trp-P, BP, DAB, aniline and other several chemicals in the presence of S-9 mix. More recently, Umezawa *et al.*¹⁵⁾ reported that, in the absence of S-9 mix, the mutagenicity of *N*-acetoxy-AAF was

greatly increased by addition of norharman or harman. In our experiment, sucrose pyrolysate had no remarkable enhancing effect on the mutagenicities of DAB and PAHs with S-9 mix and *N*-acetoxy-AAF without S-9 mix. These facts suggest that the mechanism for exhibiting the enhancing effect of sucrose pyrolysate on the mutagenicity is different from that of norharman and harman.

The presence of S-9 mix is essential for the enhancing action of sucrose pyrolysate, suggesting that interaction between the metabolic process of a mutagen and that of sucrose pyrolysate may occur.

Purification of the principle(s) which cause(s) the enhancing effect is underway in our laboratory.

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