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FEED ADDITIVES

Microbiological Production of Carotenoids. Stabilization of β-Carotene in Dried Fermentation Solids

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Storage tests were conducted on stabilization of carotene produced intracellularly by mated cultures of the mold, *Blakeslea trispora*. Addition of 0.25% of Santoquin either to the medium during fermentation or to the dried product effectively stabilized carotene. Replacement of white grease with vegetable oil in the fermentation medium resulted in a more stable product in nonprotected solids. Effective stabilization was also achieved by suspending dried mycelium in vegetable oil, by storage under inert atmospheres, or in high vacuum. Addition of a chelating agent or incorporation of dried mycelium in gelatin or casein did not increase stability.

Carotenoid pigments in natural products used in animal and poultry feeds as a source of vitamin A and xanthophyll need to be stabilized. Current methods and materials, used with only limited success are: refrigeration, storage under inert atmospheres, control of moisture, and addition of antioxidants and animal fats or vegetable oils (*11*). The intracellular β-carotene of microorganisms intended for use as provitamin A in animal feeds, like β-carotene from other sources, must be protected against oxidation when exposed to air. No published information was found on stabilization of carotenoid pigments in microorganisms. The present investigation was concerned with the stabilization of β-carotene produced intracellularly by mating of appropriate heterothallic strains of a member of the order Mucorales, *Blakeslea trispora* (*2–4*).

Materials and Methods

Two strains of *Blakeslea trispora*, NRRL 2456 (+ mating type) and NRRL 2457 (− mating type) were utilized for all experiments. Inocula were produced in 500-ml. Erlenmeyer flasks containing 150 ml. of the following medium: acid-hydrolyzed corn, 2.3%; acid-hydrolyzed soybean meal, 4.7%; thiamine hydrochloride, 1.0 mg. per liter; sodium hydroxide to pH 6.2. The flasks were sterilized for 30 minutes at 121° C., inoculated with pieces of agar containing mycelium from 5- to 6-day-old potato-dextrose agar slants of each type, and then incubated for 2 days on a rotary shaker operating at 200 r.p.m. Two flasks of culture, one of each mating type, usually containing well dispersed growth, were combined as inocula for experimental media. A 10-ml. aliquot was used to inoculate each 100 ml. of fermentation medium. This medium had the same composition as that used for production of the inoculum, but in addition contained 0.12% of nonionic detergent (Triton X-100), 4% of cottonseed oil, and 0.1% of β-ionone; the latter compound was added 2 days after initiation of fermentation. After 4 days of additional incubation on a rotary shaker at 28° C., the mycelium was steamed for 10 minutes to destroy oxidative enzymes and then was recovered by filtration. Solids were dried in a vacuum oven at 50° to 55° C., ground in a Wiley mill equipped with a 20-mesh screen, and stored in 20-gram aliquots in 125-ml. Erlenmeyer flasks loosely stoppered with cotton.

Each antioxidant was dissolved in 3 ml. of diethyl ether and then poured over the ground solids. The antioxidant was distributed by trituration with mortar and pestle, while the ether was driven off. Only antioxidants of potential use in feeds were tested.

β-Carotene was determined by previously described methods (*2*).

Results

In a series of experiments eight antioxidants were evaluated for their ability to stabilize carotenoid pigments contained in the crude, dried fermentation solids. Data from storage tests at 28° C. following addition of 0.25% (w./w.) of a variety of antioxidants are shown in Table I. At a concentration of 0.25%,

Table I. Effect of Antioxidants on Carotene Stability in Fermentation Solids Stored at 28° C.

Antioxidant, 0.25% (w./w.)	Storage, Weeks 1	2	3	4	5	6	7	8	9	10	11	12
	Carotene Remaining, %											
Comparison of Various Compounds												
Control[a]	52	26	12	..	..	..	..	..	..	..	..	..
Santoquin	95	87	81	78	72	67	63	59	54	49	44	39
BHA[b]	94	77	72	66	59	52	46	41	36	31	26	24
BHT[c]	93	79	70	61	54	48	41	34	29	24	22	21
Tenox II[d]	88	65	43	32	27	26	11	10	9	..	5	..
Tenox VI[d]	92	78	75	59	54	48	43	37	32	26	21	19
Octyl gallate	82	57	45	39	34	24	26	21	17	..	15	..
Propyl gallate	87	65	50	37	34	38	22	20	17	..	14	..
Lauryl gallate	83	73	50	36	29	26	20	19	17	..	16	..
Combination of Antioxidants												
Control[e]	3	0	..	..	..	..						
Santoquin	92	92	81	67	58	49						
BHT	78	76	50	46	34	31						
Tenox II	67	61	52	37	29	26						
Propyl gallate	63	61	53	38	17	12						
BHT + Santoquin	88	85	65	61	49	49						
Tenox II + Santoquin	81	61	55	52	49	43						
Tenox II + BHT	82	70	52	46	34	35						
Propyl gallate + Santoquin	100	85	58	57	53	49						

[a] Initial average concentration: 5700 μg. carotene/gram dry solids.
[b] Butylated hydroxyanisole.
[c] Butylated hydroxytoluene.
[d] Mixture of antioxidants (Tennessee Eastman Corp., Kingsport, Tenn.).
[e] Initial average concentration: 3280 μg. carotene/gram dry solids. Mycelium grown in medium containing choice white grease.

Table II. Effect of Increasing Concentrations of Antioxidant on Carotene Stability in Fermentation Solids Stored at 28° C.

Storage, Weeks	Santoquin, % Control[a]	0.06	0.12	0.25	0.50	1.0
	Carotene Remaining, %					
1	65	92	84	92	84	87
2	37	77	71	82	81	76
3	17	..	69	76	72	72
4	15	66	69	71	67	71
5	0	58	62	68	64	69
6	..	50	55	66	62	68
7	..	46	52	60	58	62
8	..	42	48	54	54	56
9	..	39	44	49	51	50
10	..	34	40	45	46	47
11	..	30	35	42	42	44
12	..	28	32	35	37	40

[a] Initial average concentration: 5700 μg. carotene/gram dry solids.

Santoquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline, Monsanto Chemical Co.) was superior to other antioxidants tested; a half-life of 9 to 10 weeks was recorded. Good protection was also given by butylated hydroxyanisole, butylated hydroxytoluene, and Tenox VI. Superiority of Santoquin over 53 other antioxidants for the stabilization of β-carotene in dehydrated alfalfa was also noted by Thompson (*13*) and by Siedler and coworkers (*12*) for the stabilization of vitamin A and β-carotene in feeds containing antioxidant-treated animal fats.

Incorporation of increasing concentrations of Santoquin from 0.06 to 1.0% (w./w.) gave only slight increases in stability (Table II). A half life of 6 weeks resulted when 0.06% of antioxidant was added and of 9 weeks for 0.25 to 1.0%.

Approximately 25.7 grams of dried, crude fermentation solids containing 180 mg. of β-carotene, when added to 100 pounds of poultry feed, give the desired level of 300,000 International Units of vitamin A activity. Addition of 0.25% of Santoquin to 25.7 grams of fermentation solids results in a final concentration of only 1.4 p.p.m. of antioxidant in 100 pounds of feed. This percentage is well below the 150 p.p.m. permitted in dehydrated forage crops by the Food, Drug and Cosmetic Act (*7*).

Guilbert (*8*) observed that storage temperature was a major factor governing loss of carotene in alfalfa hay and meal and that the rate of loss was approximately doubled for every 10° C. rise in temperature. In the present investigation, an increase in rate of carotene destruction was also noted when crude, dried solids were stored at increasing temperatures (Figure 1). When Santoquin was added to the crude product, carotene destruction continued to increase with rising temperatures, but not as rapidly as in the unprotected product. Carotene destruction in antioxidant-treated material tended to decrease with time.

Throughout the experiments, carotene loss in both controls and antioxidant-treated material was independent of the initial pigment concentration (Figure 2). A direct correlation exists between rate of carotene destruction in unprotected material and the type of lipide used in fermentation. Mycelium grown in media containing vegetable oil loses its carotene content less rapidly (10 to 35% loss in 1 week) than does mycelium grown on choice white grease (80 to 90% in 1 week). Varying the concentration of either lipide in the medium from 3 to 5% did not affect loss rates.

Because it is desirable to avoid extra steps and unnecessary handling of mycelium, Santoquin or other antioxidant was added directly to the fermentation medium after 2 days of incubation at a concentration of 0.25% (w./w.) based on a predicted yield of 5% solids. After recovery, the mycelium was washed with anhydrous ethyl alcohol to remove adhering antioxidant. Data in Figure 3 show that addition of antioxidant during fermentation is as effective in stabilizing carotene in the final dried product as addition directly to the dried solids. The effective stabilization achieved, despite washing of the mycelium, indicates that Santoquin, an oil-

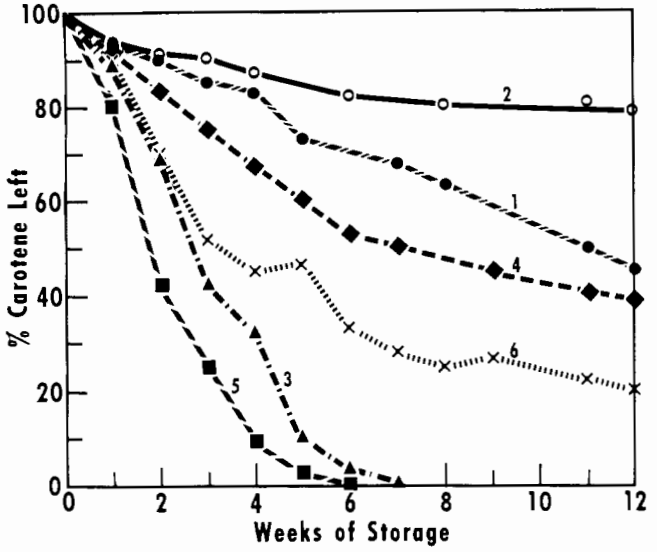

Figure 1. Effect of temperature and antioxidant on storage stability of carotene in dried fermentation solids

Test 1. Control, stored at 10° C.
2. 0.25% Santoquin added, 10° C.
3. Control, 28° C.
4. 0.25% Santoquin added, 28° C.
5. Control, 27° C.
6. 0.25% Santoquin added, 37° C.
Initial carotene concentration: 6000 μg./gram

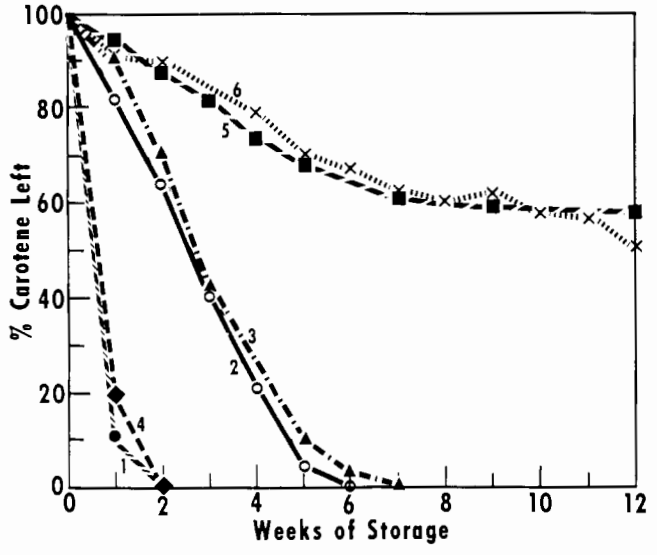

Figure 2. Effect of initial carotene concentration and type of lipide on rate of carotene loss at 28° C. storage

Test No.	Initial Concn., μg./Gram	
1	3100	Controls
2	5400	
3	6000	
4	8700	
5	4600	0.25% Santoquin added to dried solids
6	8700	

Tests 1, 4, 6. Choice white grease in fermentation medium
Tests 2, 3, 5. Vegetable oil in fermentation medium

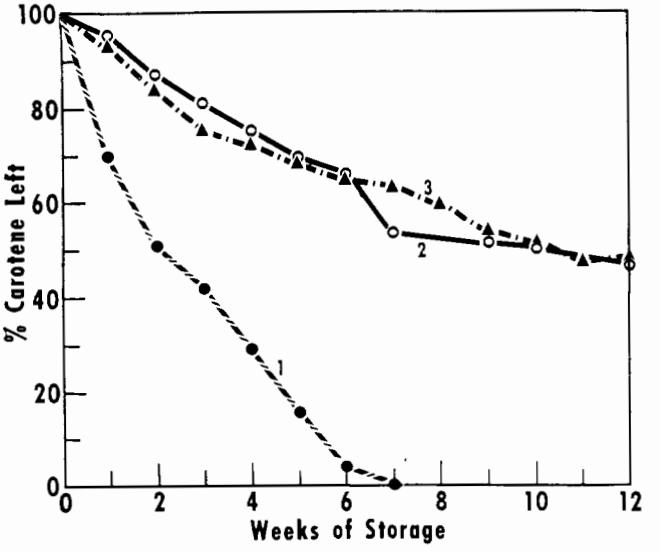

Figure 3. Effect of adding antioxidant during or after fermentation on carotene stability at 28° C.

Test 1. Control
2. 0.25% Santoquin added to dried fermentation solids
3. 0.25% Santoquin added during fermentation
Initial carotene concentration: 4700 μg./gram

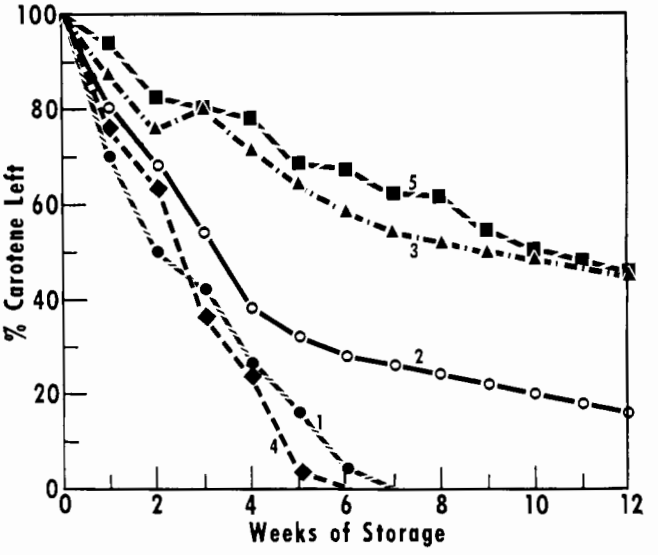

Figure 4. Effect of a chelating agent on carotene stability in dried fermentation solids stored at 28° C.

Test 1. Control
2. Versene added 1 hour before harvest
3. Versene and Santoquin added to fermentation after 48 hours incubation
4. Versene added to dried fermentation solids
5. Santoquin added to fermentation after 48 hours' incubation
Initial average carotene concentration: 5000 μg./gram

miscible compound, is taken up intracellularly during fermentation. Analyses show the mycelium to be composed of approximately 50% lipide. This lipide serves as a reservoir both for carotene, which is homogeneously distributed, and for antioxidant. Analyses of extracted lipides by paper chromatography (*6*) show that Santoquin was present. Butylated hydroxyanisole, when added during fermentation, could also be detected in the lipide fraction.

Some antioxidants have been reported to act synergistically with other compounds to give increased activity (*5, 13*). In this work three antioxidants were studied. Each of these was added at 0.12% concentration plus an equal concentration of Santoquin to the carotenoid-containing product; results were compared to those obtained with 0.25%

Table III. Stability of Carotene in Fermentation Solids Stored under Various Gases or in Vacuum for 16 Weeks at 28° C.

		Carotene Concn., μg./G.		
	Antioxidant[a]	Initial	Final[b]	Carotene Remaining, %
Air	−	4200	0	0
Air	+	4600	600	13
CO_2	−	4200	3800	91
CO_2	+	4600	4300	94
N_2	−	4200	3900	93
N_2	+	4600	4000	86
Vacuum	−	4200	4100	98
Vacuum	+	4600	4600	100

[a] 0.25% Santoquin added during fermentation.
[b] Samples stored at 28° C. for 16 weeks.

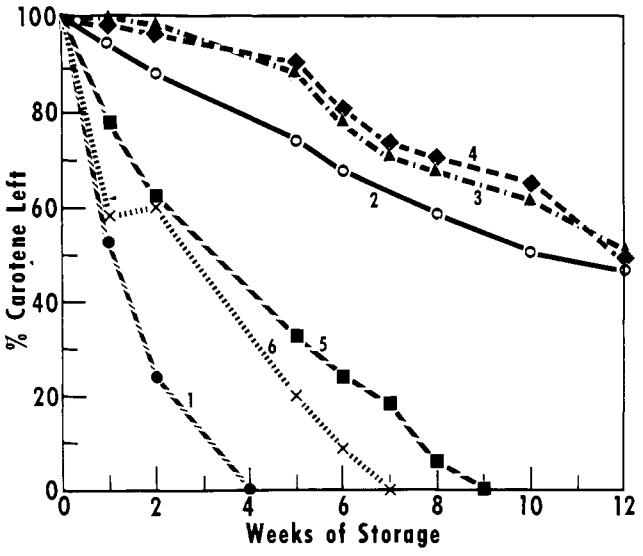

Figure 5. Effect of various lipides on carotene loss in dried fermentation solids stored at 28° C.

Test 1. Control: 7000 μg./gram initial concn.
2. Solids + 0.25% Santoquin
3. Solids + oil (1:2)
4. Solids + oil (1:2) + 0.25% Santoquin
5. Solids + brominated vegetable oil (1:2)
6. Solids + lard (1:2)

of antioxidant used alone (Table I). No advantage was gained from the use of combined antioxidants.

Heavy metals are known to catalyze the oxidation of some unsaturated compounds. To investigate the effect of a chelating agent, Versene (ethylenediamine tetrasodium acetate) was added at 0.5% concentration (w./w.) just prior to the end of fermentation and also directly to the dried product. Direct addition to the solids of 0.5% of Versene dissolved in cellosolve was not effective in stabilizing β-carotene (Figure 4). Slightly greater stability resulted when Versene was added to the fermentation medium just prior to harvest, probably because less effective chelation is possible by direct addition to a dried product. Addition of both Versene and Santoquin to the fermentation medium did not increase carotene stability in the dried product over that obtained with Santoquin alone.

Inert substances were added to the dried fermentation solids to serve as mechanical barriers to oxygen since autoxidation probably represents the principal reaction in the breakdown of carotene. To 120 grams of ground product was added 48 grams of gelatin in 300 ml. of hot water. The mixture was homogenized in a Waring Blendor with solid carbon dioxide present to provide an inert atmosphere. Santoquin (0.25%, w./v.) was added to an aliquot of the homogenate. Both aliquots, treated and nontreated, were dried in vacuum at 50° to 55° C., then ground in a Wiley mill equipped with a 20-mesh screen. In a similar experiment, formaldehyde-treated casein was substituted for gelatin (*1*). No advantage accrued by adding the above inert materials to the fermentation product.

Mitchell and his coworkers (*9*) observed that carotene in alfalfa meal could be stabilized against oxidative deterioration during storage by addition of 5% of vegetable oil to the meal followed by heating to 100° C. for 1 hour. Further improvement was achieved (*10*) by addition of 0.02% of Santoquin. Heating possibly caused greater penetration of oil and antioxidant into the meal. In the present study, addition of 5% of Wesson oil to the final product followed by heating at 100° C. for 1 hour did not improve storage stability probably because of the high percentage of lipide already present. However, a mixture of one part dried product to two parts of Wesson oil (w./w.) resulted in greatly increased stability (Figure 5). Adding Santoquin to this oil suspension did not give further protection. Lipides more saturated than vegetable oil seemed less effective.

Carotene destruction in dried alfalfa has been successfully reduced by replacing oxygen in storage installations with inert gas produced by incomplete combustion of natural gas (*11*). Similar results were obtained when 3-gram samples of fermentation product were stored in sealed test tubes that had been repeatedly flushed with carbon dioxide or nitrogen or placed under vacuum. After 4 months of storage at 28° C., 86 to 100% stability resulted in stored samples (Table III). Because of the difficulty in obtaining exact replication of analyses, the 100% figure should be interpreted as indicative rather than absolute.

Discussion

The mechanisms by which carotene in biological materials is oxidized during storage are not completely known. Blanching of the mycelium did not prevent loss of carotene during storage, indicating that destruction is primarily nonenzymatic. Retardation of carotene destruction by antioxidants, particularly Santoquin, indicates autoxidation to be the primary reaction. However, it is not clear why increasing concentrations of antioxidant do not afford complete stabilization.

That heavy metals may play a role in carotene destruction is shown by the slight protective action of Versene. Thompson (*13*) did not obtain similar results when he sprayed alfalfa with chelating agents possibly because the metal-inactivator did not penetrate to the site of carotene destruction. A similar lack of protection was noted when dry material was treated.

Vegetable oil in fermentation media and in suspending menstrua was more efficient in stabilizing carotene than were more highly saturated lipides. The reason for the greater efficiency of vegetable oil is not known. Maximum stability of carotene in dried mycelium might be achieved by storage of mycelium–vegetable oil suspensions under

inert atmosphere. Further advantage may result from the addition of antioxidant during fermentation.

Acknowledgment

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OXALATE IN BEER

The Rapid Enzymatic Determination of Oxalate in Wort and Beer

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A rapid manometric procedure for determination of oxalate in wort and beer employing the enzyme oxalic decarboxylase is described whereby oxalic acid is quantitatively decarboxylated to formic acid and carbon dioxide. Fluoride and cyanide were required as inhibitors in the presence of carbohydrate. Levels of fluoride and cyanide are critical. Excess of either alters the pH, causing low or negative results. Recovery studies were performed and average recovery was found to be 100.9% in wort, 100.2% in beer, 102% in deionized beer, and 99.8% in water. Good correlation was found between the enzymatic procedure and the precipitation-titration method.

A RAPID and accurate method for quantitative determination of oxalic acid in beer and wort has long been required. Burger and Becker (*4*) reported that sediment formed in beer on prolonged storage was in part calcium oxalate. Brenner (*3*) stated that this haze- and sediment-forming material may be a cause of gushing. Burger and Becker (*5*) mentioned oxalate as one cause for nonbiological type haze formed in old beer. In 1956, Burger, Glenister, and Becker (*6*) showed definitely that the oxalate to calcium ratio in beer is a measure of the tendency of that beer to gush.

Usual methods for quantitative determination of oxalates involve their precipitation as calcium salt. This salt may be converted into the oxide and weighed or titrated with standard acid (*4*). Yarbro and Simpson (*9*) titrated precipitated calcium oxalate with standard potassium permanganate. Permanganate titration gives an indistinct end point and high results due to interaction of permanganate with occluded organic materials in the precipitate (*6*). A 40-hour precipitation period in the cold is required (*6*) and a correction factor for the solubility of calcium oxalate must be applied (*1, 2*).

Shimazono and Hayaishi (*7*) first suggested use of oxalic decarboxylase as an analytical tool. It was used by us in developing the method described herewith, which employs the enzyme oxalic decarboxylase. Oxalic acid is quantitatively converted by this enzyme to formic acid and CO_2 according to the reaction:

$$HOOC—COOH \rightarrow HCOOH + CO_2$$

The CO_2 evolved was measured manometrically.

Experimental

Reagents. Potassium citrate buffer, 0.2*M*, pH 3.2.

Oxalic decarboxylase, prepared according to the procedure of Shimazono and Hayaishi (*7*).

Oxalic acid, 10 μmoles per ml.

Sodium fluoride, saturated aqueous solution.

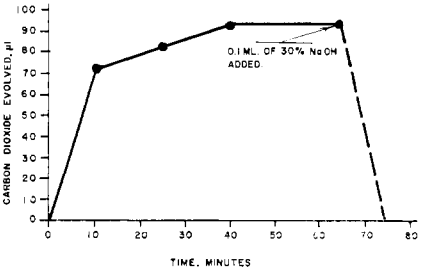

Figure 1. Rate of oxalic acid decomposition by 100 units of oxalic decarboxylase

Potassium cyanide, 10% aqueous solution.

Equipment. Warburg respirometer with single arm vessels (either plugged or vented).

Procedure. In the main compartment of a Warburg vessel are placed 3 ml. of sample (beer is first degassed), 0.1 ml. of sodium fluoride, 0.1 ml. of potassium cyanide, and 0.6 ml. of citrate buffer. One hundred units of oxalic decarboxylase dissolved in 0.2 ml. of

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