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In Vitro Fatty Acid Synthesis and Complex Lipid Metabolism in the Cyanobacterium *Anabaena variabilis*[1]

I. SOME CHARACTERISTICS OF FATTY ACID SYNTHESIS

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ABSTRACT

***In vitro* fatty acid synthesis was examined in crude cell extracts, soluble fractions, and 80% $(NH_4)_2SO_4$ fractions from *Anabaena variabilis* M3. Fatty acid synthesis was absolutely dependent upon acyl carrier protein and required NADPH and NADH. Moreover, fatty acid synthesis and elongation occurred in the cytoplasm of the cell. The major fatty acid products were palmitic acid (16:0) and stearic acid (18:0). Of considerable interest, both stearoyl-acyl carrier protein and stearoyl-coenzyme A desaturases were not detected in any of the fractions from *A. variabilis*. The similarities and differences in fatty acid synthesis between *A. variabilis* and higher plant tissues are discussed with respect to the endosymbiotic theory of chloroplast evolution.**

The cyanobacteria occupy a unique position between prokaryotes and photosynthetic eukaryotes in the evolution of photosynthetic organisms. Photosynthetic prokaryotes similar to extant cyanobacteria may have been the endosymbiont precursors of chloroplasts of higher plant cells and biochemical and physiological similarities have been found to support this relationship (15, 17).

The membrane glycerolipids of *Anabaena variabilis*, a cyanobacterium, are identical to those of other O_2 evolving photosynthetic organisms. These lipids include MGDG[3], DGDG, PG, and SL (17, 31). In addition to these four lipids, *A. variabilis* contains a small amount of MGlcDG (6) which, by epimerization of the C_4-OH group of glucose, gives rise to MGDG (6, 23) and, subsequently, DGDG (23). Because MGlcDG is also found in nonphotosynthetic bacteria—*e.g. Streptococcus faecalis* (19)—there may be possible biochemical link between *A. variabilis* and nonphotosynthetic prokaryotes.

The most abundant fatty acids in *A. variabilis* (grown at 38°C) are palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1), and linoleic acid (18:2) (22, 24), unlike higher plant leaf tissues whose major fatty acid is linolenic acid (18:3). In *A. variabilis*, as in higher plant tissues (see 12), decreasing the temperature of growth causes an increase in the proportion of C_{18} unsaturated fatty acids, particularly 18:3, esterified to the membrane glycerolipids (22, 24). The mechanism controlling the level of unsaturated fatty acids in higher plants and in the blue-green algae seems to be similar.

In view of the proposed evolutionary relationship between cyanobacteria and the chloroplasts of higher plant cells, an investigation was undertaken to characterize the fatty acid synthesis, elongation, and desaturation systems of *A. variabilis*. The results presented here represent a first step toward an examination of the localization and kinetics of the various enzymes involved in complex lipid formation in these advanced prokaryotic organisms.

MATERIALS AND METHODS

Algal Culture. Cells of *Anabaena variabilis* M3 were a generous gift from Dr. J. C. Meeks of the Department of Bacteriology, University of California, Davis. *A. variabilis* was grown on one-eighth strength Allen and Arnon (2) liquid medium supplemented with $NaNO_3$ (5 mM) and KNO_3 (5 mM). The cultures were aerated by shaking and maintained under a 16 h:8 h light:dark regime (66 $\mu E/s \cdot m^2$) at 38°C.

Chemicals. All chemicals were obtained from Sigma, and of the highest purity available, unless otherwise stated. The source of radiochemicals and specific activities are identified at the appropriate procedural descriptions. *Escherichia coli* ACP (30% pure) was provided by CalBiochem.

Preparation of Cell Extracts. Cells from mid- to late-exponential phase were harvested by centrifugation (6,000*g*, 10 min), washed with buffer, recentrifuged and suspended in the appropriate buffer (see below) containing 1 mM EDTA (2 mg/ml packed cells). The cyanobacterial suspension was incubated at RT with lysozyme chloride (1 mg/ml packed cells, 1 h), and then the cells were broken by vortexing (3 min) with washed glass beads (0.45–0.55 mm diameter; 1:1, v/v). The homogenate was filtered through glass wool and centrifuged (1,000*g*, 1 min) to remove whole cells. This supernatant ('crude cell extract') was used in enzyme assays. A soluble fraction and a membrane fraction were prepared from the crude cell extract by centrifugation of 90,000*g* (30 min). The pellet ('membrane fraction') was washed once with the appropriate buffer, recentrifuged (90,000*g*, 20 min), and resuspended in buffer. An 80% $(NH_4)_2SO_4$ protein precipitate was obtained by centrifuging the crude cell extract (13,000*g*, 20 min) and bringing the supernatant to 80% saturation with $(NH_4)_2SO_4$, centrifuging (13,000*g*, 20

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[3] Abbreviations: MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; PG, phosphatidyl glycerol; SL, the plant sulpholipid; MGlcDG, monoglucosyl diacylglycerol; PC, phosphatidyl choline; ACP, acyl carrier protein; RT, room temperature.

min), and resuspending the precipitate in a minimum of buffer.

Fatty Acid Synthesis and Elongation. Fatty acid synthesis activity and elongation of various thioesters of fatty acids were assayed according to the method of Jaworski *et al.* (9) in 0.1 M Tricine (pH 8.1). The elongation reaction was started by addition of 0.25 to 0.60 nmol of the appropriate fatty acid thioester: [1-^{14}C]myristoyl-ACP (55 mCi/mmol), [1-^{14}C]palmitoyl-ACP (55 mCi/mmol), [1-^{14}C]stearoyl-ACP (51.5 mCi/mmol), [1-^{14}C]palmitoyl-CoA (59.6 mCi/mmol; New England Nuclear) or [1-^{14}C]stearoyl-CoA (55 mCi/mmol; New England Nuclear). (The ACP thioesters were synthesized from the appropriate ^{14}C-fatty acid (New England Nuclear) and *E. coli* ACP and purified according to the method of Rock and Garwin [20]. No free fatty acids were detected in the purified fatty acid ACP thioesters synthesized.) After a 15-min incubation at RT, the reaction was stopped with 100 μl 60% KOH, and the reaction products were analyzed.

Cerulenin. The effects of different concentration of cerulenin (see text) were observed on fatty acid synthesis and elongation reactions. The assays were performed as described above. Cerulenin was prepared according to Packter and Stumpf (18).

Fatty Acid Desaturation. Stearoyl-ACP desaturase was assayed essentially according to the method described by Jaworski and Stumpf (8) and McKeon and Stumpf (16) using 0.05 M Pipes buffer (pH 6.0).

Fatty Acid Analysis. The stopped reaction mixtures were saponified by heating at 70 to 80°C, 30 min. After cooling to RT, 100 μl of 20% H_2SO_4 were added to acidify the mixture. Palmitic and stearic acids or stearic and oleic acids (10 μl, 10 mg each/ml) were added as carrier. The free fatty acids were extracted with petroleum ether or chloroform (3 × 2 ml) and the solvent was removed with a stream of N_2. An aliquot of the free fatty acids dissolved in a small volume of heptane was counted in a liquid scintillation counter (Beckman LS-230) with 10 ml scintillation fluid (Amersham PCS:xylene, 2/1, v/v). The remainder of the free fatty acids were methylated with diazomethane (11). The methyl esters were analyzed by radio-GLC using a column packed with 10% DEGS (80–100 mesh Supelcoport; Supelco) at 175°C in a Varian model 920. The reaction products of fatty acid desaturation were alternatively analyzed by silver nitrate (5%, w/w) TLC using benzene as solvent.

Protein. Protein concentration was estimated by the method of Bradford (4).

RESULTS

The data presented in Figure 1 show the cofactor requirements for *de novo* fatty acid synthesis by an $(NH_4)_2SO_4$ precipitate (see also Table I) of a crude cell extract of *A. variabilis*. Clearly, the *de novo* system is completely dependent on the presence of ACP.

Figure 1 also indicates that both reduced pyridine nucleotides, NADH and NADPH, are required for maximal fatty acid synthesis. The preferred electron donor for *de novo* synthesis was NADPH. Fatty acid synthesis in the presence of NADPH alone ('minus NADH,' Fig. 1) was greater than in the presence of NADH alone ('minus NADPH,' Fig. 1), being 23% and 2%, respectively, of the complete reaction which contained both NADH and NADPH, after 60 min. These results indicate, therefore, that one of the two reductases is NADPH specific while the second reductase can employ either NADH or less effectively NADPH. In safflower, β-ketoacyl-ACP reductase utilizes NADPH and there are two enoyl-ACP reductases, one utilizing specifically NADH while the second can employ either NADH or NADPH (27).

Omission of acetyl-CoA, a primer for fatty acid synthesis, significantly lowered the amount of [^{14}C]malonyl-CoA incorporated into fatty acids (Fig. 1). Presumably, slow decarboxylation of the added [2-^{14}C]malonyl-CoA provided sufficient acetyl-CoA to allow a low rate of fatty acid synthesis in the $(NH_4)_2SO_4$ fraction.

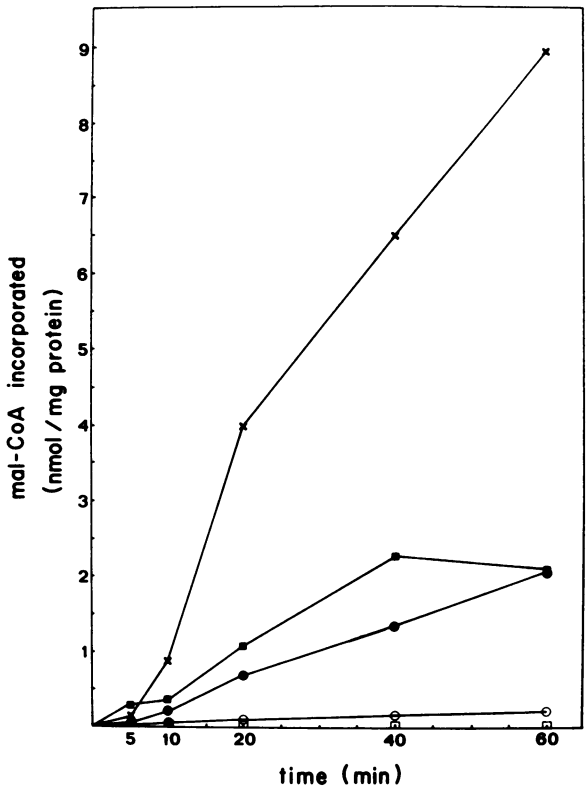

FIG. 1. *In vitro* cofactor requirements for *de novo* fatty acid synthesis in *Anabaena variabilis*. A $(NH_4)_2SO_4$ precipitate was obtained from a crude cell extract. Complete reaction, (×); minus ACP, (□); minus acetyl-CoA, (■); minus NADH, (●); minus NADPH, (○).

Investigation of the cofactor requirements for fatty acid synthesis using crude cell extracts showed similar results (data not shown).

The product of *in vitro* fatty acid synthesis in different preparations of *A. variabilis* are shown on Table I.

The major ^{14}C-fatty acids formed by the unmodified crude cell extract and without additional cofactors (ATP or DTT) were myristic (14:0) and palmitic acids (16:0). Minor ^{14}C-fatty acids were lauric (12:0) and stearic acids (18:0). Addition of ATP to the incubation medium resulted in a 66% decrease in fatty acid synthesis from [2-^{14}C]malonyl-CoA and altered the proportions of the individual fatty acids synthesized, decreasing the proportion of 14:0 and 16:0, and increasing slightly the proportion of 12:0 and 18:0 formed. However, the most dramatic changes were observed when DTT was added to the reaction mixture. There was an increase in the incorporation of total radioactivity compared to the reaction without DTT (*i.e.* 'complete') and a shift in the products from 14:0 and 16:0 (in the complete reaction) to 16:0 which made up 49.8% of all fatty acids formed. The addition of DTT also increased the proportion of 18:0 formed. (The increased fatty acid synthesis and shift toward longer fatty acids caused by DTT has also been observed for *Allium porrum* microsomes [V. Agrawal, R. Lessire, and P. K. Stumpf, unpublished].) The addition of both ATP and DTT to the crude extract tended to shift the formation of ^{14}C-fatty acids to products observed when ATP and DTT were omitted (Table I). The mechanisms of action of these components are not known.

Experiments with a soluble fraction from *A. variabilis* showed essentially the same results with respect to total synthesis and distribution of radioactivity among fatty acids as in the experiments using the crude cell extract. The responses to added ATP

Table I. *Products of In Vitro Fatty Acid Synthesis in Anabaena variabilis*

The conditions for the reactions are described in "Materials and Methods." The concentration of ATP or DTT in the incubation mixtures was 1 mM each. The final volume of $(NH_4)_2SO_4$ precipitate reactions was 0.1 ml. The reaction was initiated with 2.5 nmol [2-^{14}C]malonyl-CoA.

	Amount Incorporated	Distribution of Radioactivity			
		12:0	14:0	16:0	18:0
	dpm mg^{-1} protein $\times 10^{-4}$	%			
Crude cell extract, incubated 60 min					
Complete	6.44 ± 0.32	19.0	44.2	33.2	9.3
+ ATP	2.19 ± 0.08	24.4	39.8	23.7	13.2
+ DTT	9.98 ± 0.38	6.6	27.7	49.8	16.0
+ ATP + DTT	3.44 ± 0.37	10.4	33.6	42.7	13.4
Soluble fraction, incubated 60 min					
Complete	2.29 ± 0.14	19.8	40.4	30.6	8.9
+ ATP	1.38 ± 0.62	—[a]	—	—	—
+ DTT	6.53 ± 0.97	10.8	24.8	44.4	20.2
+ ATP + DTT	2.42 ± 0.21	15.3	33.8	38.3	12.6
$(NH_4)_2SO_4$ precipitate, incubated 30 min[b]					
Complete	11.0	3.6	13.1	42.3	40.9
+ ATP	9.67	10.9	25.4	36.6	27.2
+ DTT	19.2	0	11.7	34.1	54.2
+ ATP + DTT	13.3	0	10.9	36.1	53.0

[a] Not determined.

[b] Experiment performed once.

and DTT were identical (Table I). On the other hand, the experiments with membrane fractions (data not shown) produced very inconsistent results among duplicate samples in both fatty acid synthesis (incorporation of radioactivity) and in fatty acids products formed. In some experiments, no fatty acids were formed. These results in conjunction with the consistency of the results obtained from experiments using the soluble fraction and the results using the crude cell extract suggested that fatty acid synthesis was localized in the soluble cytoplasm (cytosol) of *A. variabilis*.

An attempt to improve incorporation of radioactivity into the fatty acids in order to observe possible synthesis of unsaturated fatty acids *in vitro* was made by concentrating the proteins of the crude cell extract with $(NH_4)_2SO_4$. Experiments using an 80% $(NH_4)_2SO_4$ precipitate showed that there was a significant increase in synthesis of fatty acids. Additional fatty acid products did not accumulate. No unsaturated fatty acids were formed, but the major fatty acids formed were 16:0 and 18:0 (Table I). The addition of ATP inhibited incorporation of radioactivity by the $(NH_4)_2SO_4$ protein precipitate, and DTT increased incorporation of radioactivity. The proportion of 18:0 was also increased by DTT as was observed with the crude cell extract and with the soluble fraction (see above).

In plastids of higher plant tissues, the fatty acid synthetase produces 16:0 as the major end product and 16:0 is elongated to form 18:0 (21, 28). Desaturation of 18:0-ACP forms 18:1 which is transferred to extra-chloroplastic PC. The elongase and desaturase enzyme systems could be assayed separately from fatty acid synthesis and the elongase ($C_{16:0} \rightarrow C_{18:0}$) can be distinguished from fatty acid synthetase ($C_2 \rightarrow C_{16:0}$) by their different responses to cerulenin (9, 18, 26, 28).

Figure 2 shows the effect of different concentrations of cerulenin on *de novo* fatty acid synthesis and 16:0-ACP elongation

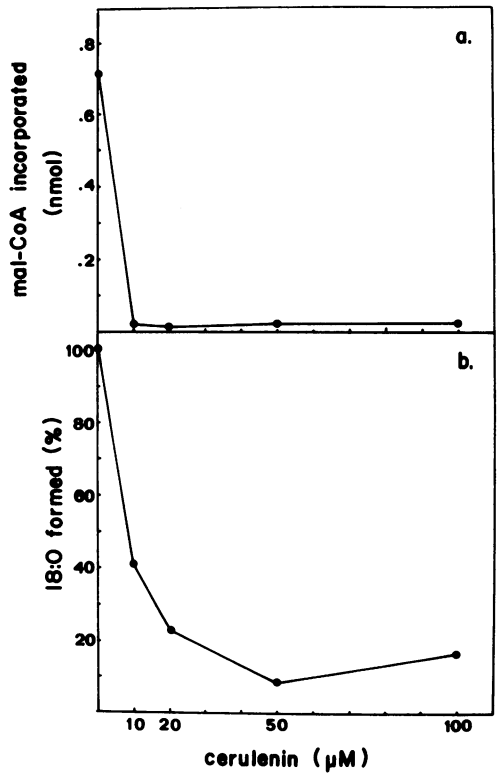

FIG. 2. Effect of cerulenin on (a) *in novo* fatty acid synthesis and (b) 16:0-ACP elongation in a crude cell extract from *Anabaena variabilis*.

Table II. *Elongation of Acyl Thioesters by Cell Preparations of Anabaena variabilis*

The conditions for the assays are described in "Materials and Methods." The amount of *E. coli* ACP added to the reaction containing CoA thioesters of fatty acids was 50 μg.

		Distribution of Radioactivity		
		14:0	16:0	18:0
			%	
Crude extract	[1-^{14}C]myristoyl-ACP	63.5	32.1	4.3
	[1-^{14}C]palmitoyl-ACP		78.2	21.8
	[1-^{14}C]palmitoyl-CoA		100	0
	[1-^{14}C]palmitoyl-CoA + ACP		100	0
	[1-^{14}C]stearoyl-ACP			100
	[1-^{14}C]stearoyl-CoA			100
	[1-^{14}C]stearoyl-CoA + ACP			100
Soluble fraction	[1-^{14}C]palmitoyl-ACP		92.0	7.9
Membrane fraction	[1-^{14}C]palmitoyl-ACP		100	0

in a crude cell extract from *A. variabilis*. The data indicate that increasing concentrations of cerulenin up to 100 μM inhibited both fatty acid synthesis and 16:0-ACP elongation. However, although there was almost complete inhibition (greater than 95%) of fatty acid synthesis activity at 10 μM cerulenin, there was only a 60% inhibition of 16:0-ACP elongation. Increasing cerulenin concentrations caused a gradual decrease in the elongation of 16:0-ACP. Complete inhibition of 16:0-ACP elongation was not observed even at the highest concentration (100 μM) of cerulenin used. These results are in agreement with the observations in higher plant tissues (9, 18, 25, 27). Previous data (25, 27) clearly has shown that the site of cerulenin inhibition is the

Table III. *Survey of Stearoyl Thioester Desaturases*

The assay conditions are described in "Materials and Methods." For *A. variabilis*, desaturase was also assayed under all conditions of pH, with NADH, NADPH, and stearoyl-CoA in addition to stearoyl-ACP.

	Stearoyl-ACP	Stearoyl-CoA
	pmol 18:1 produced/mg protein·min	
Maturing *Carthamus tinctorius* (safflower) seeds	13.3	
Spinacia oleracea (spinach) chloroplasts	36.7	
Chlamydomonas moewensii	0.17	
Chlorella pyrenoidosa	0.16	
Scenedesmus obliquus	0.19	
Anabaena variabilis	0	0
Nostoc sp.	0	

irreversible binding to β-ketoacyl-ACP synthetase I and a weaker binding to β-ketoacyl-ACP synthetase II, the enzyme involved in the specific condensation of malonyl-ACP to palmitoyl-ACP to form β-ketostearoyl-ACP.

Various CoA and ACP thioesters were tested as substrates for elongation (Table II). The elongation reaction in *A. variabilis* appeared to be specific for 16:0-ACP and was localized in the soluble fraction of the cells. The product of 16:0-ACP elongation was 18:0-ACP as in higher plant tissues (Lem and Stumpf, unpublished). [^{14}C]Myristoyl-ACP could also be elongated to form 16:0 and 18:0. Lower ACP homologues were not tested.

Of particular interest was the lack of elongation of 18:0-ACP, 16:0-CoA, or 18:0-CoA alone or in the presence of ACP (Table II). Thus, the absence of fatty acids longer than 18 carbon atoms in *A. variabilis* can be explained by the specificity of the FAS enzymes being limited to the formation of fatty acids up to 18 carbon atoms. The results were particularly revealing for 16:0-CoA since it indicated that no acyl-CoA:ACP transacylase was active in the crude cell extract. There appeared to be no 'CoA track' for fatty acid synthesis in this cyanobacterium; moreover, no acyl-CoA thioesterases or synthetases were observed in crude cell preparations (Lem and Stumpf, unpublished).

The Monoenoic Desaturation Reaction. Crude cell extracts from *A. variabilis* were not able to desaturate 18:0-ACP to form 18:1 (Table III). Desaturase was assayed in *A. variabilis* using different combinations of pH, electron donor (NADH, NADPH), and with both stearoyl-CoA and stearoyl-ACP substrates; no conversion to oleate was found. The possibility that an inhibitor in the crude cell extract may prevent 18:0 desaturation was tested by observing the effect of *A. variabilis* crude extracts on safflower stearoyl-ACP desaturase. Inhibition of safflower stearoyl-ACP desaturation was not observed (data not shown).

A survey of extracts from green algae and higher plant tissues indicated that under the conditions employed in these assays, stearoyl-ACP desaturation was present in spinach chloroplasts and the three green algae tested, *Chamydomonas moewensii, Chlorella* pyrenoidosa, *Scenedesmus obliquus*. The other cyanobacterium tested, a species of *Nostoc*, did not have stearoyl-ACP desaturase activity either, which suggested that the absence of the higher plant Δ^9 desaturase may extend to all cyanobacteria.

DISCUSSION

Fatty acid synthesis and elongation in the cyanobacterium *A. variabilis* appears to be identical to fatty acid synthesis in higher plant chloroplasts, being a ACP, NADPH, and NADH-dependent soluble system (21, 28, 29). The major product of fatty acid synthesis *in vitro* is 16:0-ACP which can be elongated to form 18:0-ACP (Fig. 2) (Tables II and III). Al'araji and Walton (1) found that the major fatty acid product formed from ^{14}C-acetate incorporation into disrupted whole cells of *Anabaena cylindrica*, and the 20,000*g* supernatant fraction, was 18:0. Using [2-^{14}C] malonyl-CoA, they were also able to find 18:1 synthesis (13.4% of total fatty acid radioactivity) in the 20,000*g* supernatant fraction. We were unable to find 18:1 synthesis under any conditions, and stearoyl-ACP desaturase activity was not detected. (This latter observation has been independently confirmed by J. Jaworski [personal communication].) The absence of 18:0-ACP desaturase represents a major departure from the pathway of C_{18} unsaturated fatty acid synthesis in higher plant tissues.

In chloroplasts, 18:1-ACP is the major initial product of fatty acid synthesis; it is then hydrolyzed by oleoyl-ACP hydrolase and the free fatty acid is transported to the outer envelope, to be converted to oleoyl-CoA. This substrate is employed for further modification in the cytosolic compartment (3, 12, 28). The unsaturated C_{18} fatty acids, 18:2 and 18:3, are returned to the chloroplast to become esterified to MGDG, and further desaturation of 18:2 to form the large quantities of 18:3 esterified to MGDG and DGDG occurs on MGDG (7, 14, 30). The absence of an extrachloroplastic compartment and PC in *A. variabilis* raises interesting questions of where and how 18:1 is desaturated.

The pathway of 18:0 desaturation in *A. variabilis* is not known. Sato and Murata (24) have recently proposed a mechanism for C_{18} fatty acid desaturation in *A. variabilis* based upon analyses of the pulse labeling kinetics of whole cells fed with $NaH^{14}CO_2$. In their model, desaturation of 18:0, 18:1, and 18:2 may occur in the membrane glycerolipids, MGDG, SL, PG, as well as MGlcDG. These results are provocative in that they provide still another mechanism for the insertion of a Δ^9 double bond in stearic acid. The other systems include the anaerobic mechanism of eubacteria (5), the stearoyl-ACP desaturase of higher plants (see 15), and the stearoyl-CoA desaturase of animal tissues.

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