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GLYCOLLATE METABOLISM IN PHOTOSYNTHESISING TISSUE

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SUMMARY

1. Glycollate oxidase (glycollate:oxygen oxidoreductase, EC 1.1.3.1) was not detected in chloroplasts prepared by the method of WALKER, in non-aqueous chloroplasts or in chloroplast lamellae.

2. The oxidase activity associated with some chloroplast preparations was shown to be due to adsorption from the supernatant solution on to the chloroplasts and to cytoplasmic contamination.

3. The results from experiments *in vitro* were confirmed *in vivo* by feeding of [^{14}C]glycollate to spinach leaf tips. Only a small fraction of the counts taken up was found in the chloroplasts and glycollate itself was not metabolised by non-aqueously isolated chloroplasts.

4. The possible function of glycollate in carbon and hydrogen transport is discussed.

INTRODUCTION

Glycollic acid formation has been reported during photosynthesis in unicellular algae[1–5], in higher plants[5,6] and in isolated chloroplasts[22]. While the almost stoichiometric conversion of CO_2 to glycollate at low CO_2 partial pressures reported by WARBURG AND KRIPPAHL[1] is not generally observed, there is little doubt that a large fraction of newly fixed CO_2 may be metabolised *via* glycollate and its derivatives[5,7]. A pathway from glycollate to sucrose *via* glycine and serine has been demonstrated in a variety of tissues[8–13]. This pathway can be blocked *in vivo* using an inhibitor of glycollate oxidase, when glycollate accumulates in large amounts[14].

Both phosphoglycollate phosphatase (phosphoglycollate phosphohydrolase, EC 3.1.3.18) and glyoxalate reductase (glycollate:NADP oxidoreductase)—two enzymes probably involved in glycollate synthesis—are present almost exclusively in the chloroplast[15]. Little, however, is known of the intracellular site of action of glycollate oxidase (glycollate:oxygen oxidoreductase, EC 1.1.3.1). Activity has been attributed to the chloroplast[16,17], the mitochondrion[18] and to the soluble supernatant[19,20].

Much of the reported variation in results has been due to the use of contami-

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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nated chloroplast preparations. It has been shown, for example, that after centrifugation at 1000 × *g* the sediments not only contain chloroplasts but also biochemically active mitochondria[21].

There is much speculation regarding the role of glycollate in intracellular metabolism. In the present work glycollate oxidase has been assayed in chloroplasts isolated by several different techniques. The significance of the results is discussed in relation to [^{14}C]glycollate feeding experiments *in vivo*.

METHODS

Spinach was the major source of plant material. In spring and summer it was possible to use outdoor-grown plants. In winter they were grown in the greenhouse under conditions of normal winter daylength. Only completely healthy young leaves were used, being removed from the plant not more than 15 min before the beginning of an experiment. The midribs were cut out and the leaves washed with distilled water, dried between blotting paper sheets and placed in a cold room at 4° before use.

Chloroplasts were extracted according to the method of ARNON, WHATLEY AND ALLEN[22]. The grinding medium used was 0.1 M phosphate buffer (pH 8.0) containing 0.01 M sodium isoascorbate and 0.35 M NaCl. The pellet from the first centrifugation at 1000 × *g* was washed by suspension in 0.35 M NaCl containing 0.01 M sodium isoascorbate. After the second centrifugation the pellet was resuspended in the same medium. Such a preparation was referred to as 'Arnon type' chloroplasts.

An alternative method of chloroplast isolation used was that due to WALKER[23,24]. 0.4 M sucrose in both the grinding medium and the resuspending medium was replaced by 0.35 M NaCl. Chloroplasts prepared by this method were termed 'Walker type' chloroplasts. It was shown that whilst they did not preserve their structure as long as those prepared in sucrose or sorbitol, the preparation had a majority of plastids with their outside membranes intact immediately after preparation.

Chloroplast lamellae were prepared according to LEECH[25] after the method of JAMES AND DAS[26]. The final pellet was suspended in 0.006 M Tris–HCl buffer (pH 7.5) containing 0.3 M sucrose.

Chloroplasts were isolated non-aqueously by the method of BIRD, PORTER AND STOCKING[27]. 100 mg of freeze-dried spinach leaves were homogenised in 15 ml of a carbon tetrachloride–hexane mixture, $d = 1.36$ g/cm^3. After filtration, and readjustment of the volume to 15 ml the suspension was transferred to a centrifuge tube and an equal volume of density 1.34 was layered on top. A further 2-ml volume of density 1.32 completed the gradient. After centrifugation the top layer containing the chloroplasts was removed, diluted with an equal volume of hexane and spun down at 3000 × *g*. The last traces of organic solvent were removed by evacuating the tubes in a desiccator on a water pump for 10 min. The chloroplasts appeared after this final operation as a bright green powder. The yield on a weight basis was between 4–6 % of the initial freeze-dried material. Resuspending media were as indicated in the RESULTS section. Chlorophyll was determined by the method of ARNON[28] and protein by that of LOWRY *et al.*[29].

Glycollate oxidase was partially purified by the method of ZELITCH AND OCHOA[30]. The fraction from the second ethanol precipitation was used as a source of

crude enzyme and had a specific activity of 1.3 μmoles glycollate oxidised per mg protein per h.

Fumarase (L-malate hydro-lyase, EC 4.2.1.2) activity was measured by the increase in absorbance at 240 mμ on addition of malate (PIERPOINT[19]).

[1-^{14}C]Glycollate was fed in the light *via* the petiole of spinach leaf tips as described by MIFLIN, MARKER AND WHITTINGHAM[34]. After photosynthesis for different periods of time, the leaves were removed individually with tweezers and the cut edge washed and dried. The leaves were then cut into thin strips and dropped into a flask precooled to −195° in liquid N_2. The time between removal from the trough and freezing was never more than 20 sec.

The non-aqueous technique for the isolation of chloroplasts required a minimum of 100 mg freeze-dried material. This was equivalent to about 1 g fresh weight. Thus for each separate isolation procedure at least 6 leaf tips were fed.

When sufficient leaf material had been obtained the flask was transferred from the liquid N_2 to an acetone–solid CO_2 mixture at −79°. The contents were later freeze-dried and the chloroplasts isolated as described above. The chloroplast and chloroplast-depleted cytoplasm fractions were extracted twice in boiling 90 % ethanol. The insoluble residue was further extracted in 20 % ethanol and the combined supernatants were concentrated in a rotary evaporator at a temperature not greater than 35°. Under these conditions the distillate did not become radioactive. The residue, after vacuum desiccation was taken up in a known volume of 50 % ethanol. A fraction was counted under an end window Geiger–Müller tube to give the total counts in the soluble fraction and a fraction was retained for chromatography. The original insoluble residue was transferred to a small tube, dried down in a vacuum desiccator and suspended in a known volume of 50 % ethanol. Aliquots were dried down and counted as above.

The distribution of label in the soluble fraction was determined using the method of 2-dimensional chromatography as described by PRITCHARD, GRIFFIN AND WHITTINGHAM[35].

RESULTS

Some glycollic acid oxidase was present in Arnon type chloroplasts but none was found in those prepared by the method of WALKER[23,24] (Fig. 1).

Glycollate oxidase activity was removed from Arnon type chloroplasts after passage down a glycerol–sucrose density gradient. Electron micrographs indicate that such 'purified' preparations are devoid of external membranes and stroma and that there is little mitochondrial contamination[25]. Thus cytoplasmic material originally associated with the chloroplasts is removed after centrifugation on a glycerol gradient. Were the activity to remain in the chloroplasts after 'purification' it would almost certainly be associated with the lamellae; on the other hand absence of activity can only eliminate a lamellar but not a stromal or outer membrane site of action. Results are shown in Fig. 2. It was further shown that glycollate oxidase was readily absorbed on to chloroplasts during their isolation from a tissue homogenate. Of glycollate oxidase added to the grinding medium about 20 % became adsorbed to the chloroplast fraction (Table I). About one fourth to one third of the total original chloroplastic glycollate oxidase was lost in the first washing. A further washing removed

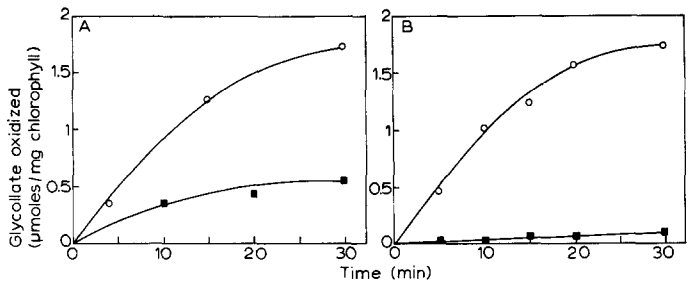

Fig. 1. Glycollate oxidase in 'Arnon' and 'Walker type' chloroplasts. The reaction mixture contained in a final vol. of 1.4 ml the following components, in μmoles: Mg^{2+}, 2.1; potassium phosphate (pH 8.0), 90; chloroplasts or supernatant (by the method of ARNON (A) or WALKER (B)) containing between 50 and 70 μg chlorophyll. The reaction was started by addition of 1 μmole sodium glycollate. 'Supernatant' refers to the solution remaining after removal of chloroplasts. Glycollate disappearance was determined colorimetrically by the method of CALKINS[32]. ■, chloroplasts; ○, supernatant.

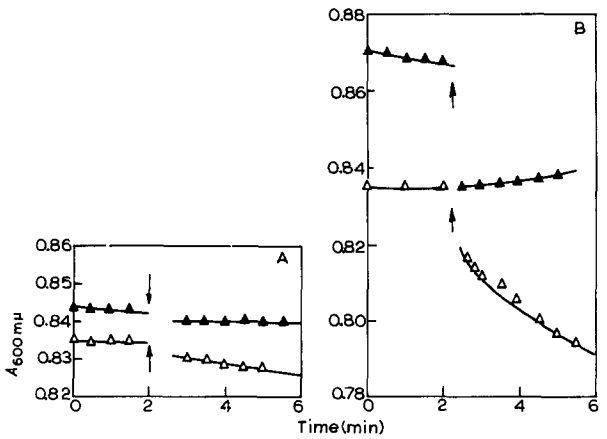

Fig. 2. Glycollate oxidase in chloroplasts from a glycerol gradient. The reaction mixture contained in a final vol. of 2.5 ml the following components, in μmoles: potassium phosphate (pH 8.0), 30; DCIP, 0.1; KCN, 10; NH_4OH, 1; FMN, 0.05; lamellae (A) or chloroplasts (B) containing 21 μg chlorophyll. The reaction was started by addition of 20 μmoles sodium glycollate (indicated by arrows). The decrease in absorbance was measured at 600 mμ in a Hilger spectrophotometer (ZELITCH AND OCHOA[30]). △, lamellae or chloroplasts; ▲, control (no added glycollate).

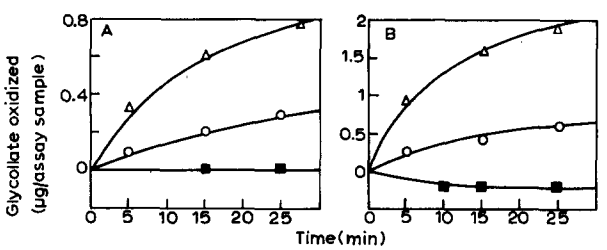

Fig. 3. Retention of glycollate oxidase by chloroplasts. Method as for Table I except that 5 ml glycollate oxidase were used (B). Glycollate disappearance was measured by the method of CALKINS[32]. Chloroplasts from both homogenates were washed in decreasing volumes of grinding medium in order to keep the chlorophyll concentration constant at 50 μg chlorophyll per reaction mixture. The control (A) had no added oxidase. △, unwashed; ○, once washed; ■, twice washed chloroplasts.

the remaining activity. In this way a chloroplast preparation free from oxidase activity could be obtained (Fig. 3). Again, removal of chloroplasts from a tissue homogenate did not alter significantly the specific activity of the supernatant (Fig. 4).

The concentration of cytoplasmic material can be arranged to increase in going down a non-aqueous density gradient. If glycollate oxidase was not chloroplastic then the activity should increase in going down a gradient. (For convenience all non-chloroplastic material will be termed 'cytoplasm'.)

Glycollate oxidase was assayed in successive fractions down a non-aqueous

TABLE I

GLYCOLLATE OXIDASE ADSORPTION ON CHLOROPLASTS

Alternate right- and left-hand halves were combined from 24 g spinach leaves. The chloroplasts were isolated from both sets of halves by the method of ARNON, WHATLEY AND ALLEN[22] except that the grinding medium for one set contained 8.0 ml of glycollate oxidase (prepared as described under METHODS). Chlorophyll concentrations of each of the four preparations differed slightly and were corrected to an arbitrary value of 10 μg chlorophyll per assay sample. Assays as described under Fig. 1.

Fraction	*Specific activity (μmoles glycollate oxidised/mg protein/h)*	
	Homogenate + oxidase	*Control homogenate*
Chloroplasts	7.1	3.1
Supernatant	22	9.4

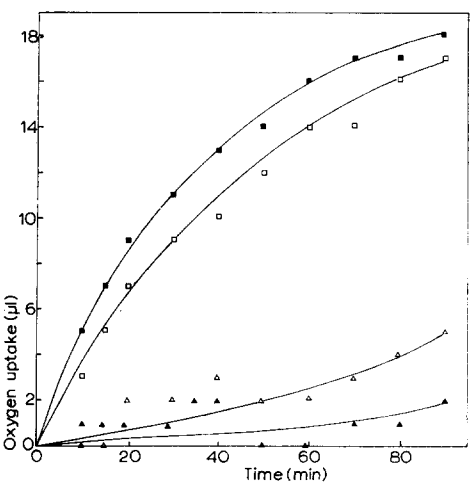

Fig. 4. Comparison of glycollate oxidase activity in whole tissue with that of the supernatant remaining after removal of chloroplasts by the method of ARNON. 100 mg freeze dried spinach leaves were homogenised in 15 ml 0.35 M NaCl in 0.1 M phosphate buffer (pH 8.0) containing 0.0025 M Mg^{2+}. This suspension was termed whole tissue. Chloroplasts were resuspended in 5 ml 0.1 M phosphate buffer (pH 8.0). Glycollate oxidase was assayed manometrically (CLAGETT, TOLBERT AND BURRIS[31]) in chloroplasts, whole tissue and supernatant. The flasks contained in a final vol. of 3 ml the following components, in μmoles: Tris buffer (pH 8.0), 75; Mg^{2+}, 4.5; 1 ml solution to be assayed and the reaction started by the addition of 20 μM glycollate from the side arm. ■, whole tissue containing 120 μg chlorophyll/ml; □, supernatant containing 70 μg chlorophyll/ml; △, chloroplasts containing 140 μg/ml; ▲, whole tissue control (no added glycollate).

TABLE II

GLYCOLLATE OXIDASE IN FRACTIONS FROM A NON-AQUEOUS DENSITY GRADIENT

Fractions were withdrawn using a Pasteur pipette. Each was diluted with an equal volume of hexane and centrifuged for 10 min at 3000 × *g*. The supernatant was poured off and the pellets dried in a vacuum desiccator for 10 min. They were then suspended in 0.05 M phosphate buffer (pH 8.0) and 0.1-ml samples assayed for oxidase activity, as described under Fig. 1.

Fraction	*Glycollate (μg) oxidised/fraction*
1	0
2	0
3	3.2
4	7.0
5	48.0

TABLE III

FUMARASE IN DIFFERENT CHLOROPLAST PREPARATIONS

Assays as described under METHODS.

Preparation	*Fumarase 240 mμ (units of absorbance/mg chlorophyll/h)*
Non-aqueous chloroplasts	0
Non-aqueous supernatant	13.1
Broken chloroplasts	0
'Arnon type' chloroplasts	
(1) in 0.4 M sucrose	5.2
(2) in 0.35 M NaCl	4.1

TABLE IV

DISTRIBUTION OF COUNTS AFTER [1-^{14}C]GLYCOLLATE FEEDING TO WHOLE TISSUE

Leaf segments were fed with sodium [1-^{14}C]glycollate and chloroplasts subsequently isolated by the non-aqueous technique. For details see under METHODS. The percentage of the total counts taken up present in chloroplasts was determined by the method of HEBER, PON AND HEBER[39].

Time of feeding (min)	*% chloroplast protein in total protein*	*Spec. activity (counts/min/mg protein)*						*Yield of isolated plastids***	*% of total counts absorbed present in chloroplasts*	
		Chloroplasts		*Intact tissue*	*Chloroplast-depleted tissue*	*Cytoplasm*				
		*A**	*B***			*A**	*B***		*A**	*B***
5	29.3	1847	1489	2330	1944	2527	2043	8.1	23.0	14.6
15	39	2458	1965	3325	3493	3880	3862	41	28.8	17.0
30	41	2281	1525	3666	3734	4631	4621	11.4	25.5	12.8

* A, results uncorrected for cytoplasmic contamination.

** B, corrected values assuming that no more than 10% of the total cytoplasmic activity is associated with the chloroplasts.

*** In % of total chlorophyll content of tissue.

density gradient (Table II). The top two fractions, the upper of which had a relatively high chlorophyll content, had negligible activity. Fractions 3 and 4 had an increasing activity which reached a maximum in Fraction 5. Fumarase activity was taken as a measure of cytoplasmic activity and the distribution of this enzyme in the various fractions followed closely that of glycollic acid oxidase (Table III).

Table IV shows the distribution within the cell of counts taken up when leaf segments were fed with [1-^{14}C]glycollate. From 12.8–17 % of the total counts taken up were found in the chloroplasts of which less than 3 % was present as free glycollate (Table V). [$^{14}C_2$]Glycollate was not metabolised by chloroplasts isolated by the non-aqueous technique.

TABLE V

DISTRIBUTION OF GLYCOLLATE BETWEEN CHLOROPLASTS AND CYTOPLASM

For details see Table IV and METHODS.

Time of feeding (min)	*% chloroplast protein in total protein*	*Spec. activity (counts/min/mg protein)*						*Yield of isolated chloroplasts***	*% of total coun taken up presen as free glycollat in chloroplasts*	
		Chloroplasts		*Intact tissue*	*Chloroplast-depleted tissue*	*Cytoplasm*				
		*A**	*B*			*A*	*B*		*A*	*B***
5	29.3	695	217	2330	1944	2997	2445	8.1	8.6	2.7
15	39	700	68	3325	3493	5003	4533	41	8.2	0.76

* Specific activity of chloroplasts expressed here as counts/min in free glycollate/mg protein.
** These figures obtained assuming not more than 5 % of the total cytoplasmic glycollate is associated with the chloroplasts.
*** As % of total chlorophyll of tissue.

TABLE VI

DISTRIBUTION OF COMPOUNDS LABELLED FOUND IN CHLOROPLASTS AND CHLOROPLAST-DEPLETED CYTOPLASM

For details see under METHODS. Data for each experiment given as % of total metabolised in the fraction; that for total metabolised as 10^3 counts/min.

Compound	*Chloroplasts*		*Chloroplast-depleted cytoplasm*	
Time of feeding	*5 min*	*15 min*	*5 min*	*15 min*
Glycollate	—	—	—	—
Glycine	26.4	13.7	33.5	15.1
Serine	37.6	51.8	33.0	47.5
Sucrose	7.6	22.3	—	18.6
Malate	8.0	4.6	3.9	3.6
Sugar phosphates	20.4	7.5	29.5	15.1
Insolubles	—	—	—	—
Total metabolised	1.85	1.87	4.98	5.9

DISCUSSION

TOLBERT AND COHAN[8] and DELAVAN AND BENSON[36] have concluded that glycollic acid oxidase is associated with the chloroplasts. While the present work has

shown that some glycollate oxidase is associated with Arnon-type chloroplasts, it has demonstrated that this activity is to be attributed to cytoplasmic contamination and enzyme adsorption on to the chloroplasts.

Fumarase is generally considered to be a mitochondrial enzyme; yet while some activity was present in Arnon-type chloroplasts, none was detected in those prepared according to the method of WALKER[23,24] or by the non-aqueous technique. Part, if not all, of the glycollate oxidase of chloroplasts may be due to contaminating mitochondria.

In contrast to Arnon-type chloroplasts, 'intact' chloroplasts appear to be homogeneous in the light microscope. While some mitochondria are visible in electron micrographs[25] no enzyme activity associated with these could be detected. Such chloroplast preparations are capable of high rates of $^{14}CO_2$ fixation (WALKER[37]) suggesting that loss of protein during isolation is small. No glycollate oxidase was found in chloroplasts (50–90 % intact) prepared by this method. Neither was any activity associated with free lamellar systems prepared from a glycerol gradient. However, ZELITCH AND BARBER[17] have reported that malate and glycollate oxidation by isolated chloroplasts was inhibited by addition of 20 % glycerol to the suspension. Thus, while the lack of activity in lamellae may be due to extraction of soluble enzymes, the possibility of some inactivation cannot be ruled out.

More than 25 % of the adsorbed glycollate oxidase could be removed on one washing and the remainder in a second washing. However, ZELITCH AND BARBER[17] using chloroplasts prepared by a method similar to that of ARNON, WHATLEY AND ALLEN[22] found that the addition of glycollate to the washed preparation always gave a marked rise in O_2 uptake. This oxidase was not removed by as many as 4 washings and it was concluded that some enzyme was present in the chloroplast. It may well be that the lower pH (7.2–7.5) combined with a grinding medium containing mannitol–borate buffer and EDTA used by ZELITCH favoured enzyme adsorption and retention by chloroplasts.

On the other hand these results confirm those of PIERPOINT[19,20] using 'intact' chloroplasts from a sucrose gradient, in which it was concluded that the enzyme was associated neither with chloroplasts nor with mitochondria.

On a non-aqueous density gradient as the density increased, and therefore, the proportion of cytoplasmic components to chloroplasts also increased, so the oxidase activity showed a pronounced increase. The lightest fractions, *i.e.* those containing most chloroplasts, were inactive; the oxidase activity was associated with cytoplasm rather than with chloroplasts. The question of cytoplasmic contamination of the chloroplast fraction need not be considered since no oxidase activity was present in that fraction. There was no reason to believe that any specific inhibition of the enzyme occurred in the chloroplasts since all fractions experienced the same isolation procedure; furthermore added oxidase was not inhibited by the chloroplasts.

Spinach leaf tips readily metabolised externally fed [1-^{14}C]glycollate *via* glycine, serine and sucrose. Compounds labelled were very similar to those found by MIFLIN, MARKER AND WHITTINGHAM[34] (Table VI).

The results suggest that glyoxalate produced by cytoplasmic oxidation of glycollate enters the chloroplast where it is metabolised to glycine, serine, *etc.* Other compounds formed from glycollate in the cytoplasm may also enter the plastid.

Glycollate has been implicated both in carbon and in hydrogen transport from

the plastid to the cytoplasm[33,30]. Under conditions of low partial pressure of CO_2 glycollate may function as a carbon carrier, replacing sugar phosphates which may be more important under other conditions.

On the other hand, since glyoxalate reductase is confined to the chloroplast and glycollate oxidase to the cytoplasm, the possibility of a glycollate–glyoxalate oxidoreduction cycle operating across the chloroplast membrane is also to be considered. In such a system the pyridine nucleotide of the chloroplast is reduced in light *via* ferredoxin. Glyoxalate within the chloroplast will then be reduced to glycollate by NADPH and glyoxalate reductase. If the glycollic acid is excreted from the chloroplast it could be oxidised externally to glyoxalate and then again re-enter the chloroplast. This mechanism first proposed by ZELITCH AND OCHOA[30] has some of the features of a hydrogen-transport system. The transport of 2 hydrogen atoms from the chloroplast to O_2 in the cytoplasm requires two intermediate hydrogen carriers. The pyridine nucleotide as the initial acceptor can remain in the chloroplast while glycollate and glyoxalate act as 'shuttle' substrates between chloroplasts and cytoplasm.

A similar process may operate in liver mitochondria with α-hydroxybutyrate and acetoacetate as 'shuttle' substrates (DEVLIN AND BEDELL[38]).

It may be noted that (a) glyoxalate reductase has an equilibrium greatly in favour of glycollate formation[40]; (b) the reverse reaction with glycollate oxidase has not been demonstrated[30]; (c) glycollate movement across the membrane appears to be uni-directional.

Whilst these points are not conclusive, in as far as conversion of glycollate to glycine could still take place within the chloroplast by coupling the conversion of glycollate to glyoxalate with the formation of glycine from glyoxalate, such a mechanism would fail to give a rationale to the observed localisation of the enzyme systems involved.

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