Phytotoxic Action of Paraquat on the Photosynthetic Apparatus

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Treatment of microalgae *(Bumilleriopsis)* with paraquat (1,1-dimethyl-4,4-dipyridylium dichloride) under culture conditions in the light for 20 or 160 h leads to light-induced oxygen uptake and more or less severe chlorophyll bleaching, which is accompanied by formation of malondialdehyde. The ratio of chlorophyll to packed chloroplast volume remains about the same as that of the control, presumably indicating destruction of membranes concurrently with pigments. Unrelated to retardation of growth, degree of bleaching or to the formation of malondialdehyde quite a uniform degree of inactivation ($\approx 50\%$) of partial redox reactions is observed in the region of photosystem II and I except for the terminal part of photosystem I (pigment 700 $\rightarrow$ $NADP^+$).

The action of paraquat in the cell centers primarily on the photosynthetic membrane system and redox chain.

Introduction

Dipyridylium salts, like paraquat (1,1-dimethyl-4,4′dipyridylium dichloride) can be reduced by photosystem I [1–3] or by dark processes like NADPH oxidation via suitable reductases (*e. g.* [4, 5]) leading to cationic radicals [6, 7]. Since the phytotoxic effects of the dipydridyls are exerted mainly in the presence of oxygen [7,9] it is assumed that the reaction products of the cationic radical with oxygen are the phytotoxic species (see [10, 11] for review). Apparently, the superoxide anion (O_2^-) originates first (see [12], with refs. therein) and may subsequently react with other cell components. Further, two O_2^- anions combine to yield hydrogen peroxide (either in the presence of superoxide dismutase or not) which may form hydroxyl radicals ($\cdot OH$) by reaction with O_2^-, particularly when suitable metals or enzyme systems are present [11, 13]. Also the generation of reactive singlet oxygen ($^1O_2$) was reported by reaction of O_2^- with peroxide [14] or by the nonenzymic dismutation of two superoxides (see [13, 15, 16] for possible reaction mechanisms). Consequently it is assumed that besides hydrogen peroxide itself [10] superoxide anion, hydroxyl radical and singlet oxygen are the phytotoxic species coming up in the course of dipyridyl herbicide application, although the occurrence of the latter two has yet to be substantiated. They may cause, for example, peroxidation of unsaturated (membrane) lipids by a radical chain reaction [11, 17, 18] which can be detected by excretion of malondialdehyde from the cell [19].

The first effect after application of dipyridyls to plants in the light is the inhibition of photosynthetic gas exchange and cessation of growth [20]. The herbicide prevents the photosynthetic reduction of $NADP^+$. However, this first effect is accompanied by damage to plasmalemma and tonoplast membranes, and by subsequent deterioration of other cell structures while breakdown of pigments and thylakoids was reported to occur at a later stage [21–23]. In principle, the same situation is observed with the green alga *Chlorella* [24].

Very little is known about a change of photosynthetic electron transport activity under the influence of dipyridyls. The only short report is from Harris and Dodge [22] who measured a pretty rapid inactivation of ferricyanide-mediated Hill reaction after a short-term treatment of flax cotyledons with 0.1 mM paraquat.

Therefore, this study was undertaken to provide more information on photosynthetic electron trans-

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Abbreviations: asc, sodium ascorbate; Chl, chlorophyll a; DAD, 3,6-diaminodurene 2,3,5,6-tetramethyl-p-phenylenediamine (from Fluka-Roth, Karlsruhe); DBMIB, 2,5-dibromo-3-ethyl-6-isopropyl-p-benzoquinone (from Dr. Trebst, Bochum); DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (from Riedel de Haën, Hannover); DPC, 1,5-diphenylcarbazide (from Eastman-Kodak, Rochester, NY, USA); Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid); MDA, malondialdehyde; Mes, 2-(N-morpholino)-ethanesulfonic acid; MV, methylviologen (=paraquat, 1,1′-dimethyl-4,4′-dipyridylium dichloride); SiMo, silicomolybdic acid (from Climax Molybdenum Co., New York, NY, USA); TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine (from Merck AG, Darmstadt); Tricine, N-tris(hydroxymethyl)-methylglycine.

port activity after exposure of the intact cell to a herbicidal dipyridylium salt. In cell free systems, partial photosynthetic redox reactions after a short-term cultivation (18 to 20 h) were compared with those observed after long-term cultivation (160 h) in the presence of paraquat in order to find differences due to the duration of herbicide application. The xanthophycean alga *Bumilleriopsis* was used in these experiments, since (1) the herbicide dosage to microalgae is easily reproducible, (2) this investigation is focused on dipyridyl effects to the photosynthetic apparatus only, (3) isolated chloroplasts from this species are suitable for measurement of electron transport reactions, and (4) this alga is also used for investigations on other herbicidal substances whose effects should eventually be compared (see *e. g.* [25]).

Materials and Methods

Bumilleriopsis filiformis Vischer (a Xanthophycea from the stock collection of the first author) was grown for 3 days up to $1.5-2\times10^6$ cells/ml (chlorophyll per cell $1.1-1.5\times10^{-6}$ µg) in sterile autotrophic medium at 22 – 23 °C. Then, the cultures were adjusted to certain cell densities when indicated and further grown in the presence of paraquat. In the experiments of Tables I and II the cells were grown at 8000 lux in a Kniese growth apparatus [26] for short-term herbicide treatment, whereas 5000 lux were applied in the long-term treatments (comp. Table I) and cultivation was done in 2-l Fernbach flasks on a shaker [27]. In both experiments, the light source was a bank of fluorescent lamps as described [28]. Sometimes, the nutrient medium was changed for that of Miller and Fogg (given in [26]), but the results were about the same as with the medium according to [27]. – Determination of cellular parameters and photosynthetic activities of chloroplasts were performed as reported [28]. Reaction time with all cell-free assays did not exceed 2 min. Details on the composition of the assays are given in the legend of Table II. Coupled chloroplasts (free of envelope, type C) were prepared according to [29] unless noted otherwise. For determination of packed chloroplast volume 50 to 100 µl of the chloroplast suspension (containing 0.075 to 0.15 mg of chlorophyll) were filled up to 2 ml with the reaction medium mentioned in the legend of Table II and centrifuged for 20 min at $1200\times g$ in a graduated micro centrifuge tube (Resistance, 80 µl capacity). The average error was ±10%. Malondialdehyde excretion was determined after [30].

Chemicals

All buffers and reagents for assays were purchased either from Serva, Heidelberg, or Sigma, München, unless mentioned otherwise in the list of abbreviations. DAD and DCMU were re-crystallized twice before use. Chemicals, pro analysi, for algae cultures were from Merck AG, Darmstadt.

Results and Discussion

Fig. 1 indicates that paraquat rapidly decreases photosynthetic oxygen evolution giving rise to a light-induced oxygen uptake. Dark oxygen uptake was not substantially affected with any of the three concentrations used (it decreased somewhat when the application time was at least twice as long). These data confirm earlier reports with *Chlorella* [24] indicating a strong effect of paraquat concentration on the decrease of oxygen evolution. The curves point out that for our purposes the herbicide concentration applied has to be rather low to ensure a period of apparent, albeit reduced photosynthesis and some growth and cell development during paraquat treatment. All three concentrations used here eventually cause a light-induced oxygen uptake of about the same rate, close to the value of dark respiration. During exposure of cells to paraquat a substantial accumulation of the herbicide inside the cells did not occur. (This was determined by applying the culture medium again to normally grown cells.) It is assumed from these cellular data that simple electron deviation during photosynthetic electron transport (giving rise to light-induced oxygen uptake) cannot be the only action of paraquat. Otherwise the rate of oxygen uptake should at least be different between lower paraquat concentrations. This can be concluded from light-induced O_2 uptake performed with isolated chloroplasts according to Nr. 2 of Table II, which decreased markedly when the paraquat concentration was kept below 0.1 mM.

This assumption is evidenced further by Fig. 2. With 0.5 mM paraquat applied to light-exposed cultures oxygen uptake develops very rapidly and its rate can be markedly enhanced by the addition

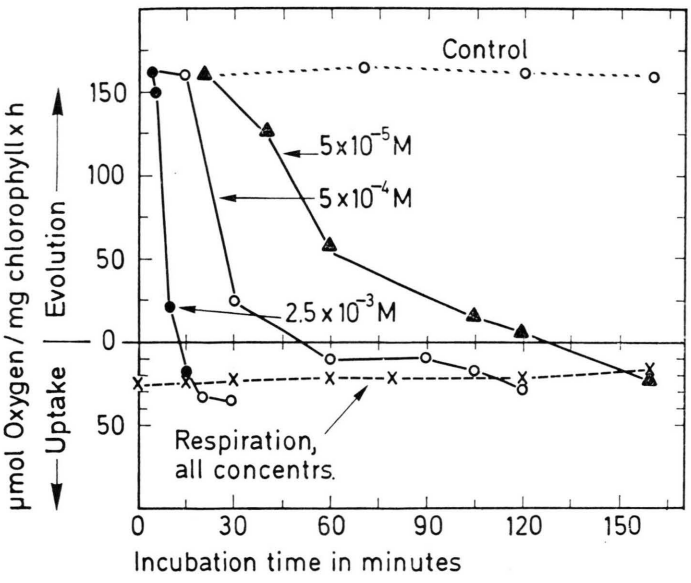

Fig. 1. Photosynthetic oxygen evolution, respiration and development of light-induced oxygen uptake of *Bumilleriopsis* cells under the influence of paraquat in the light. Chlorophyll/cell: 1.1×10^{-5} µg. Light intensity 5000 lux. 500-ml samples were exposed to paraquat in Fernbach vessels under culture conditions.

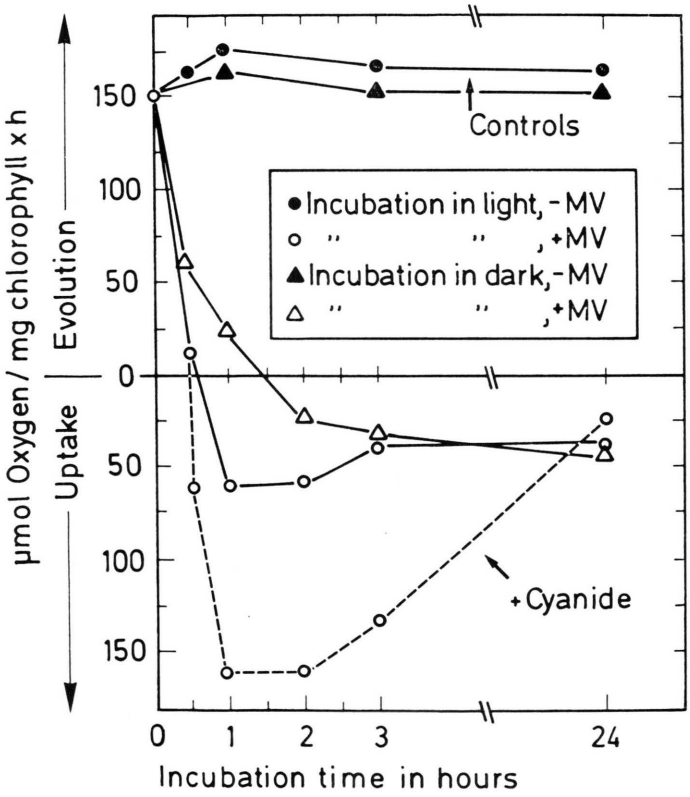

Fig. 2. Development of light-induced oxygen uptake of *Bumilleriopsis* cells in the presence of 0.5 mM paraquat. When indicated 1 mM NaCN was added 1 min before measurement of oxygen. Chlorophyll/cell: 1.2×10^{-5} µg; bleaching in the light after 24 h: −40% *vs.* control. Other details see Fig. 1.

of cyanide during measurement of photosynthesis. 1 to 2 hours after paraquat application, cyanide causes light-induced oxygen uptake of about the same numerical value as photosynthetic oxygen evolution of the control. Azide (1 mM) did the same. This is indicative of an unimpaired photosynthetic electron transport, its electrons donated from water but being accepted exclusively by the dipyridyl. Since the hydrogen peroxide then formed cannot be broken down due to the cyanide present,

a stoichiometry as expected is observed. However, after 2 hours, light-induced oxygen uptake decreases and reaches zero after approx. 24 h. The small oxygen uptake and its subsequent decrease without cyanide present during measurement was also observed by Stokes *et al.* [24], but their figures – obtained without cyanide present – do not indicate a relation of oxygen uptake to the photosynthetic electron transport capacity of the system.

In the light, incubation of cells with the herbicide favours the rapid appearence of oxygen uptake. In the dark, cells have to be exposed to the dipyridyl (e. g. 50 μM) about 10 times longer than in the light in order to exhibit the same rate of oxygen uptake. As not documented here, this is not due to a slower paraquat uptake but appears to be related to distribution processes of the herbicide in the cell (comp. [31] for translocation effects in higher plants). This problem has to be pursued in a separate investigation.

After paraquat application for 1 – 2 h no influence on electron transport capacity can be seen. This means that neither the rate of light-induced O_2 uptake with intact cells (in the presence of 1 mM cyanide) nor the O_2 uptake of the cell-free system is impaired. Under prolonged paraquat influence, however, the decrease of cellular light-induced oxygen uptake as shown in Fig. 2 is apparently due to a decrease of photosynthetic electron transport activity. In the following, this is documented in some detail.

Table I presents cellular data measured after short-term and long-term application of paraquat under culture conditions. In both sets of experiments a certain growth (which takes place at start of cultivation) is evident. Although the concentrations of paraquat used here were much lower than in the experiments of Figs. 1 or 2, a similar rate of oxygen uptake in the light was eventually observed in both experiments (No. 3). Interesting to note that the degree of chlorophyll bleaching is very different, namely -28% *vs.* control in the short-term and -69% in the long-term application (No. 2). The formation of malondialdehyde is more pronounced in the long-term experiment suggesting that chlorophyll bleaching is accompanied by breakdown of lipids.

The degree of growth retardation, MDA formation and pigment bleaching is not correlated with photosynthetic activities of the cell-free system. Furthermore, a short (20 h) or a long (160 h) exposure of cultures to paraquat did not result in differential inactivations of partial electron transport reactions. Most of the reactions were inhibited by 40 to 60% *vs.* control as documented in Table II (Nos. 2, 3). Further, we do not observe a complete inactivation of redox activity preceding pigment bleaching as was reported for ferricyanide reduction with flax cotyledon chloroplasts [22]. In our assays, the inactivation of electron transport is independent of the transport rate since the uncoupled system, being accelerated about 3 times by NH_4^+,

Table I. Photosynthetic parameters of paraquat-treated *Bumilleriopsis* cells.

		Short-term: Cultivation time: 18–20 h		Long-term: Cultivation time: 160 h	
		Control (–) paraquat	(+) 8 μM paraquat	Control (–) paraquat	(+) 0.5 μM paraquat
1) Cell density $\frac{\text{cells}}{\text{ml}} \times 10^6$	at start	1.5	1.5	0.4–0.5	0.4–0.5
	at harvest	2.8	2.2 (–21%)	6.2	1.6 (–74%)
2) $\frac{\text{nmol Chl}}{\text{cell}} \times 10^{-6}$		12.5	9.0 (–28%)	16	5.0 (–69%)
3) $\frac{\mu\text{mol } O_2}{\text{mg Chl} \times \text{h}}$	light	161	–87	129	–16
	dark	–35	–40	–21	–33
4) $\frac{\text{nmol MDA}}{\text{cell}} \times 10^{-6}$		0.50	1.0 (+100%)	0.6	1.6 (+270%)

Means of 6–8 experiments, deviation about ±20% with herbicide material and ±10% with controls. The algae were inoculated with the cell density as indicated in No. (1) and grown autotrophically with or without paraquat added to the culture medium. A (–) sign before data of No. 3 indicates oxygen uptake.

Table II. Photosynthetic redox reactions with chloroplast material isolated from paraquat-treated *Bumilleriopsis*.

	Short-term: Cultivation time: 18–20 h			Long-term: Cultivation time: 160 h		
	(−) paraquat	(+) 8 μM paraquat		(−) paraquat	(+) 0.5 μM paraquat	
1) μg Chl per μl packed chloroplast volume	8.3	9.0		8.1	8.5	
2) $H_2O \rightarrow$ MV	60	23	−62%	40	20	−50%
+ DPC	60	28	−53%	41	21	−49%
+ NH_4Cl	180	71	−60%	110	40	−63%
3) $H_2O \rightarrow NADP^+$						
+ NH_4Cl	242	96	−60%	190	76	−60%
4) $H_2O \rightarrow$ SiMo	270	130	−52%	230	93	−60%
+ DCMU	230	112	−51%	210	80	−62%
5) (−) DAD, asc → MV	12	20	—	10	15	—
6) DAD/asc → MV						
DAD: 0.1 mM	854	456	−47%	480	260	−46%
+ DBMIB	820	440	−46%	460	230	−50%
DAD: 0.015 mM	403	240	−40%	274	165	−40%
7) TMPD/asc → MV						
0.1 mM	566	348	−39%	337	185	−45%
0.025 mM	340	202	−40%	202	128	−37%
8) DCIP/asc → MV						
0.2 mM	274	284	+ 4%	244	276	+12%
0.1 mM	—	—		180	165	− 8%
0.05 mM	152	165	+ 8%	—	—	
9) mamm. cytochrome c → MV						
+0.1% Triton	570	595	+ 4%	—	—	
+0.2% Triton	644	600	− 7%	490	460	− 6%

Data expressed as μmol O_2 taken up (Nos. 2, 5–8); μmol O_2 evolved (No. 4) or μmol $NADP^+$ reduced (No. 3). All rates refer to mg chlorophyll and hour. Means of 6–8 expts. Cell densities and cellular parameters as in Table I.

Details of cell-free assays (see also "Methods"):
The reaction medium contained in mM unless mentioned otherwise: Hepes-NaOH, pH 7.8, 50; sorbitol, 600; $MgCl_2$, 5; K_2HPO_4, 10; *Bumilleriopsis* chloroplasts equivalent to 20–30 μg of chlorophyll/ml were added immediately before each assay. Gas phase: air. In all cases light-induced oxygen uptake was measured at the Clark electrode [28] unless mentioned otherwise. Following additions or alterations were made (components denoted by a [+] sign in the table were present only when indicated):

to (2): MV, 0.25 mM; Na-azide, 0.25 mM DPC, 0.5 mM; NH_4Cl, 0.5 mM;

to (3): MES-NaOH buffer, pH 7.8, 50 mM instead of Hepes; sucrose, 60 mM instead of sorbitol; $NADP^+$, 0.2; *Bumilleriopsis* ferredoxin 1.8–2.0 μM; $MgCl_2$, 2; NH_4Cl, 0.5 mM; chlorophyll 8–12 μg/ml. The reduction of $NADP^+$ was determined spectrophotometrically;

to (4): Reaction medium: Hepes-NaOH, pH 8.0, 50 mM; sucrose, 60 mM; $MgCl_2$, 2; SiMo, 0.3 mg/ml; *Bumilleriopsis* chloroplast material isolated according to [27]. DCMU, 0.25–0.5 μM. Highly coupled C-type chloroplasts prepared after [29] and assayed in the general reaction medium (see above) exhibited stronger DCMU, inhibition of the $H_2O \rightarrow$ SiMo system suggesting (partial) reduction of SiMo at photosystem I;

to (5): Na-ascorbate, 0.2 M;

to (6): MV, 0.25 mM; Na-azide, 0.25 mM; DCMU, 0.25 μM; DAD+asc, 0.1 mM+1 mM (or 0.015+0.15 mM); DAD-stock solution contained 10 mM DAD dissolved in 0.01 N HCl; DBMIB, 2 μM;

to (7, 8): MV, azide, DCMU like (6); TMPD+asc, 0.1 mM+1 mM (or 0.025+0.25 mM); DCIP+asc, 0.2 mM+2 mM (or 0.1+1 mM; 0.05+0.5 mM);

to (9): Reaction mixture: Tricine-NaOH, pH 8.0, 80 mM; MV, azide, DCMU like (6); mammalian cytochrome c-550 (reduced previously by sodium dithionite), 60 μM; Triton X-100 as indicated; Photooxidation of cytochrome c-550 was also determined spectrophotometrically.

exhibits the same degree of inhibition (No. 2). Since further the inactivation of light-induced $NADP^+$ reduction is of the same degree as that of the $H_2O \rightarrow MV$ system, no particular influence on the redox *proteins* of the terminal part of photosystem I is evident. Moreover, the physiological $H_2O \rightarrow NADP^+$ system indicates that no paraquat remains bound to the chloroplasts after their isolation from the herbicide-treated cells. If this were the case, $NADP^+$ reduction should have been more impaired than electron transport through *e. g.* the $H_2O \rightarrow MV$ system due to some electron deviation from the nucleotide. The water-splitting part is not limiting since diphenylcarbazide does not alleviate inactivation of electron transport (No. 2). The rate of silicomolybdate reduction (No. 4) is lowered by about 50% indicative of correspondingly minimized photosystem-II activity. SiMo is reduced close at the quencher Q thereby showing almost no DCMU inhibition [32].

Also the DAD/asc → MV and TMPD/asc → MV system (Nos. 6, 7) are affected to about the same degree by paraquat treatment.

The latter two donors mainly react after the plastoquinone site since their reaction is very little inhibited by DBMIB ($1-2\ \mu M$) which blocks linear transport ($H_2O \rightarrow MV$) to 90% and more. The reaction sequence, with e. g. reduced DAD as donor, however, strictly includes the plastidic c-type cytochrome(s) present in *Bumilleriopsis* [29, 33]. On the other hand, the DCIP/asc → MV system and mammalian cytochrome c-550 photooxidation are not influenced at all, even when the reaction conditions are adjusted to different rates by varying the concentration of *e. g.* DCIP (Nos. 8, 9 of Table II). In our system, these donors react quite close to pigment (P)700, the reduced DCIP presumably directly with the reation center chlorophyll, since we have been able to show with isolated spinach (and *Bumilleriopsis*) chloroplasts that neither their incubation with amphotericin nor KCN (leading to destruction of plastocyanin in spinach chloroplasts [33]) inhibited electron donation by reduced DCIP but did so when reduced DAD or TMPD was used (unpubl. results). – As shown further in experiment No. 5 of Table II, ascorbate does not function as direct electron donor to photosystem I of chloroplasts from normally grown or from paraquat-treated cells. This is in contrast to findings with flax chloroplasts where paraquat treatment of cotyledons apparently "opened" up the photosystem-I region allowing to feed in electrons directly by ascorbate without a mediator [34].

The data show that P700 and the reducing side of photosystem I are not affected by paraquat treatment of the cell, but apparently all partial reactions of the other regions of the photosynthetic redox chain were influenced. The same degree of inactivation of reactions using either water or reduced DAD as electron donor indicates that the overall integrity of the membrane system or of the redox chain complex, respectively, has been disturbed. Further, it is evident that an advanced lipid peroxidation – as in the long-term paraquat treatment – has no bearing on the degree of the electron transport inactivation. However, the activity of the redox chain may nevertheless be due to a small but decisive part of the membrane lipids, whose deterioration does not show up quantitatively in MDA formation.

It appears that paraquat-mediated breakdown or inactivation of membrane components is a fast process which is not followed by a chain of slower secondary effects on other photosynthetic reactions. Otherwise the short-term experiment should not have shown the same inactivation pattern as that of the long-term paraquat treatment.

When pigment bleaching is involved in herbicide attack the reliability of chlorophyll as a reference has to be checked. However, the ratios of chlorophyll to packed chloroplast volume were found constant (No. 1 of Table II) and had the same value as the controls. We tentatively assume that these determinations indicate the amount of thylakoid material present. So, our data point to an additional effect of paraquat treatment, namely an apparent breakdown of thylakoid material together with a distinct amount of pigments, leaving constant the ratio of chlorophyll to packed chloroplast volume. Consequently, chlorophyll can be taken as a suitable reference to compare activities of cell-free systems.

It should be noted that treatment of algal cells with difunon [35] or substituted pyridazinone herbicides [25, 28] results in a different effect. With certain concentrations, chlorophyll may be bleached without concurrent attack of the redox system leading to an *increase* of rates of photosynthtic redox reactions when referred to chlorophyll. Then, the rates of the cell-free systems may

be raised as much as the cellular chlorophyll content is lowered. In constrast to paraquat, these herbicides apparently act more specifically on the (plastidic) pigment inventory only.

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