

Potentiometric Cyanine Dyes Are Sensitive Probes for Mitochondria in Intact Plant Cells¹

KINETIN ENHANCES MITOCHONDRIAL FLUORESCENCE

Received for publication December 5, 1986 and in revised form May 11, 1987

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ABSTRACT

Selected fluorescent dyes were tested for uptake by mitochondria in intact cells of barley, maize, and onion. The cationic cyanine dye 3,3'-diheptyloxycarbocyanine iodide [DiOC₂(3)] accumulated in mitochondria within 15 to 30 minutes without appreciable staining of other protoplasmic constituents. The number, shape, and movement of the fluorescent mitochondria could be seen readily, and the fluorescence intensity of the mitochondria could be monitored with a microscope photometer. Fluorescence was eliminated in 1 to 5 minutes by the protonophore carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) indicating that maintenance of dye concentration was dependent on the inside-negative transmembrane potential maintained by functional mitochondria. Fluorescence of prestained mitochondria was enhanced within 5 to 10 minutes after addition of 0.1 millimolar kinetin to cells. The fluorescence in kinetin-treated cells was dissipated by CCCP. These results suggest that kinetin interacted with respiratory processes resulting in higher potential across the mitochondrial membrane.

Fluorescent probes have been applied as optical indicators of membrane potential differences in several types of cells, isolated organelles, and lipid vesicles (2, 6, 29, 30). The technique relies on potential-dependent partitioning of charged lipophilic dye molecules across the membrane. Changes in membrane potential result in changes in intensity of dye fluorescence, termed 'redistribution signals' (6).

Potentiometric dyes have been used successfully to measure changes in membrane potential of mitochondria within or isolated from cells of yeasts (15, 24), several kinds of animal cells (3, 7, 8, 13, 14, 16, 25), and recently, in cells or protoplasts of higher plants (18, 26). The potentiometric dyes most widely used for mitochondria are either derivatives of rhodamine, especially

rhodamine 123, or cyanines, dyes developed by Waggoner (30) and co-workers for use as potentiometric probes. Through the use of respiratory inhibitors and ionophores and of others means, the fluorescence intensity of these dyes has been shown to be related to mitochondrial membrane potential (2, 6, 8, 13.).

We have recently found that certain cyanine dyes gave fluorescence to mitochondria of haustoria within living barley epidermal cells infected with *Erysiphe graminis* (4). The mitochondrial fluorescence was rapidly dissipated by the protonophores CCCP² or DNP indicating that maintenance of dye concentration within the mitochondria was dependent on membrane potential. Several of the cyanines tested were observed also to stain mitochondria of host epidermal cells, which prompted us to screen various cyanines and other dyes used elsewhere as mitochondrial probes for ability to stain higher plant mitochondria. Our objectives were to learn which dyes were most effective with cells of higher plants, to learn if fluorescence of stained plant mitochondria could be monitored photometrically (as had been possible for haustorial mitochondria), and to learn if fluorescence was related to mitochondrial membrane potential. We also measured the effect of kinetin on mitochondrial fluorescence. Our interest in kinetin grew out of the fact that kinetin had interfered with development of haustoria of *E. graminis* (17). Although kinetin had no obvious effect on haustorial mitochondria, we found that it markedly enhanced the fluorescence intensity of host mitochondria.

The use of certain cyanine dyes as cytological or potentiometric stains for higher plant mitochondria was reported (18, 26) as we were completing our investigation. Our report here confirms and extends these studies and compares the effectiveness of several different dyes. We also show that mitochondrial fluorescence can be measured photometrically in individual living plant cells and that kinetin enhances that fluorescence.

EXPERIMENTAL PROCEDURE

Reagents and Solutions. Fluorescent compounds tested for their ability to act as membrane potential probes in the mitochondria of living plant cells are listed in Table I. All fluorescent compounds were from Molecular Probes (Eugene, OR 97402). All dyes were dissolved in a small volume of ethanol except D-296, which was dissolved in a small volume of DMSO (Matheson, Coleman and Bell, Cincinnati). Stock dye solutions were made at 10 μ M in potassium phosphate buffer (0.01 M, pH 6.4) and

¹ This research was supported in part by grants from the McKnight Foundation, The National Institute of General Medical Sciences (GM-19398), and the United States Department of Agriculture (85-CRCR-1-1596). Cooperative investigation of Agricultural Research Service, United States Department of Agriculture and the Minnesota Agricultural Experiment Station. Paper No. 15,060, Scientific Journal Series, Minnesota Agricultural Experiment Station. Mention of a trademark name or proprietary product does not constitute a guarantee or warranty by the United States Department of Agriculture or imply its approval to the exclusion of other products that may also be suitable.

² Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DIC, differential interference contrast; DiOC₂(3) and other dyes, see Table I.

subsequently serially diluted with buffer to the concentrations desired. Ethanol or DMSO concentrations were adjusted to 0.5% in the final solution.

Kinetin (6-furfurylamino-purine) (ICN Biochemicals, Cleveland) and zeatin (6-[4-hydroxy-3-methylbut-2-enylamino]purine) (Sigma Chemical Co.) were dissolved in a small amount of DMSO and diluted with phosphate buffer to 0.1 mM and then diluted serially to lower concentrations as required. Additional DMSO was added to all solutions as necessary to obtain 0.5%. CCCP (Sigma Chemical Co.) was dissolved with a small amount of ethanol and diluted with phosphate buffer to 1 μ M. Ethanol concentration was adjusted to 0.5% in the final solutions.

Plant Cell Preparation. Three kinds of plant cells were used in this study: epidermal cells of barley 'Algerian/4* (F14) Man. S' and onion (*Allium cepa* L. 'Downing Yellow Globe') and callus culture cells of maize (*Zea mays*, Line A-188). Barley epidermal tissues were dissected and mounted as described by Bushnell *et al.* (4) from coleoptiles grown in light (12). The tissue used was part of the inner epidermis of the tubular coleoptile. Two vascular bundles were left attached to the edges of the tissue to support it during manipulation. The tissue, with attached bundles was spread on a glass microscope slide, and one coverslip then was placed on each end of the tissue. The central uncovered part of the tissue was used for experimental treatment and observation. The tissue was incubated with distilled water placed beneath both coverslips and the tissue. Epidermal tissues of the third scale (from the outside) of onion bulbs were stripped, placed in water on glass slides, and covered with a cover slip. Maize callus cultures were newly initiated from embryos and cultured on Murashige-Skoog medium for 21 d. At this stage, callus cells were in loose clumps and could be transferred individually onto glass slides without damage. The maize cells were mounted in water on the glass slides under a coverslip.

Staining of the Mitochondria. Dye solutions were applied to mounted tissues or cells by applying 4 to 6 drops of solution at an edge of the slip covering the specimens. A piece of filter paper was placed at the other edge of the cover slip to withdraw water and to draw the dye solution underneath the coverslip. For mounted barley tissues, dye was applied to one coverslip and water was withdrawn from the other coverslip, drawing dye solution beneath the tissue that extended between the two coverslips. The tissues with dye solution were incubated in the dark at 18°C for 1 h. The dye solution was then withdrawn with a piece of filter paper. Except as noted, the dye solution was replaced with distilled water and the tissues were incubated at 18°C for another hour to reduce background staining before fluorescence was observed and measured.

Fluorescence Microscopy and Photography. Stained cells were examined with a Zeiss fluorescence photomicroscope equipped with epiillumination from a 100 W mercury arc lamp, using a BP 450-497 exciter filter, a FT 510 dichroic mirror, and an LP 526 barrier filter. Areas of interest on the slide were initially identified with an X40 objective lens under bright field, phase or DIC microscopy. Observations of fluorescence from selected cells were made with a X40 (0.7 numerical aperture [NA]) dry objective lens. Photographs were taken by using AGFA VARIO-XL 400 film with the automatic exposure control of the microscope set at ASA 1600 for fluorescence microscopy or at ASA 400 for bright field, phase, or DIC microscopy.

Quantitative Measurements of Fluorescence. Measurements of fluorescence intensity from selected cells were made with a X40 (0.67 NA) dry objective lens with an aperture that allowed fluorescence emission to be detected from a specimen disc of 20 μ m diameter. Fluorescence intensity was measured with a Zeiss O1K microscope photometer.

Fluorescence intensity was measured within 10 to 30 s after the fluorescence excitation shutter was opened, and the shutter

was closed as soon as the reading was made to minimize photo-destruction. Decrease in fluorescence was insignificant over such a short period of excitation. Three measurements were made at each time point on different fields and an average was calculated for the time point.

Individual mounts were used for quantitative studies only if mitochondria were generally fluorescent as judged by a brief observation. Fields within mounts were located with bright field microscopy to avoid edges of cells. Before fluorescence intensity was measured for a given field, the microscope was focused on mitochondria in the cytoplasmic layer near the upper surface of the cell. Mounts were always moved to the right between fields to avoid repeated readings at the same place. Successive fields were sometimes observed within a single cell and sometimes in neighboring cells.

Effect of CCCP. The effect of the protonophore, CCCP, was measured on cells prestained with DiOC₇(3). The stained cells were exposed to 1 μ M CCCP after the original fluorescence intensity had been measured. Fluorescence was measured again 1, 5, 10, 15, 20, 30, and 60 min after the CCCP was added.

Cytokinin Treatments. Kinetin and zeatin solutions were applied to specimens under coverslips in the same way as were dye solutions. Fluorescence change was determined at 5, 10, 15, 20, and 60 min after kinetin was added. Sixty min after kinetin treatment, CCCP at 1 μ M was applied and fluorescence intensities were measured.

RESULTS

The dyes tested (Table I) included a series of 10 cyanine derivatives which varied in length of alkyl side chains (R₁, Fig. 1) and in substitutions in the '7' position of the conjugated rings (R, Fig. 1). Also tested were three other cationic dyes, DASPMI, D-296, and rhodamine 123 (Table I). When applied at 1 μ M to

Table I. Properties of the Fluorescent Dyes Tested

Dye	Side Chain	Staining Properties		
		Mitochondria ^a		Cytoplasm ^b
		Without CCCP	With 1 μ M CCCP	
Cyanines^c				
DiOC ₁ (3)	methyl	+ ^d	-	+
DiOC ₂ (3)	ethyl	+	-	+
DiOC ₃ (3)	propyl	+ ^d	-	+
DiOC ₄ (3)	butyl	++ ^d	-	+
DiOC ₅ (3)	pentyl	++	-	+
DiOC ₇ (3)	heptyl	+++	-	++
DiSC ₂ (3)	ethyl	++	-	+
DiSC ₃ (3)	propyl	+	+	+
DiIC ₂ (3)	ethyl	++	-	+
DiIC ₄ (3)	butyl	++ ^d	-	+
Other				
DASPMI ^e		+++	-	+
D-296 ^f		+	-	+
Rhodamine 123		-	-	-

^a Fluorescence noted on following scale: -, no mitochondria fluorescence; +, few mitochondria fluoresce, and then weakly; ++, intermediate and variable numbers of mitochondria fluoresce, with moderate to bright intensity; +++, numerous mitochondria fluoresce brightly (as in Figs. 2-5). ^b Cytoplasmic fluorescence (other than mitochondrial) noted on following scale: -, no fluorescence; +, diffuse fluorescence. ^c Codings for cyanines follow Sims *et al.* (28) (see Fig. 1 for general structure). ^d Some unidentified spherical bodies were stained in addition to mitochondria. ^e 2-[4-Dimethylaminostyryl]-N-methylpyridinium iodide. ^f 2-(4-[Dimethylamino]phenylbutadienyl)-N-methylquinolinium iodide.

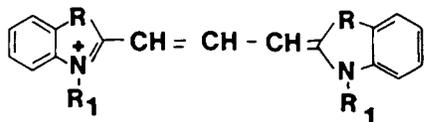


FIG. 1. Structure of the cyanine dyes. R can be O, S, or C-(CH₃)₂ (coded as 'I' in Table I). R₁, can be various lengths of alkyl chain (coded C_n in Table I).

cells of barley, maize, and onion, all the tested dyes except rhodamine 123 gave faint fluorescence to cytoplasm and at least some fluorescence to mitochondria, usually within 10 min after dye was applied (Table I). For the cyanines, the more hydrophobic the dye, the better was the mitochondrial staining. Thus, DiOC₇(3) with a heptyl side chain, gave the most intense mitochondrial fluorescence (Table I). DiOC₇(3) produced minimal fluorescence in the cytoplasmic background (Table I) and thus gave the best contrast between mitochondria and background. DASPMI also stained mitochondria but was cytotoxic even at low concentration (0.1 μM) causing mitochondria to clump within 10 min, a phenomenon observed with some other cytotoxic substances (Z Liu, unpublished data). D-296 gave only weak fluorescence to mitochondria. Rhodamine 123 gave no intracellular fluorescence even at high concentration (1 mM).

Staining Patterns and Tissue Variability in Mitochondrial Accumulation of DiOC₇(3). DiOC₇(3) stained mitochondria of living plant cells in 15 to 30 min. The dye traversed the cell wall and plasmalemma and entered cytoplasm in about 10 min, initially appearing as a faint general fluorescence in the cytoplasm. As the staining process proceeded, individual mitochondria fluoresced as bright, straight rods, as thinner filaments with one or more bends, or sometimes as spheres (Figs. 2–5). Filaments frequently became spheres, especially if cells were moved or pressed. The staining reached a maximum within an hour as judged visually. The nuclei of maize callus cells tended to be lighted by mitochondria which crowded around them (Figs. 2–3). The nuclei themselves showed little or no fluorescence (Figs. 2–5). Diffuse fluorescence was usually seen in cell wall regions. Within cells, long, thin, faintly fluorescent filaments were seen to extend in the longest dimension of the cells. These filaments often extended the entire length of the cell. They were more frequent in barley and onion epidermal cells than in corn callus cells.

Fluorescence of DiOC₇(3) in cells was subject to a moderate rate of photodestruction. About 30% of initial fluorescence was lost within 10 min if the field was kept under continuous excitation. More importantly, some individual mitochondria lost all visible fluorescence at about 10 min. For this reason, excitation of individual fields was limited to 30 to 60 s for quantitative measurements of fluorescence intensity.

Within a given cell, all the mitochondria were stained with a similar fluorescence intensity as judged visually, suggesting that the functional state of all mitochondria was uniform in a single cell. Furthermore, no obvious variations were observed among cells in a given mount. However, large variations were observed among mounts. In some, none of the mitochondria were fluorescent. Mitochondrial fluorescence appeared to be an on-off phenomenon for each preparation. The reasons for the lack of fluorescence in some mounts were not established, although care to avoid injury to cells when mounts were prepared tended to improve consistency of fluorescence. Among species, the relative fluorescence intensity was highest with onion cells, intermediate with barley cells, and lowest with maize cells. A 15% difference was observed between onion and barley, and another 5% between barley and maize. The differences were probably due to different numbers of mitochondria per unit area among the three kinds of cells (within the 314 μm² areas used for photometric measure-

ments). The highest density of mitochondria was observed with onion cells and the lowest density with maize cells. No obvious differences in brightness of individual mitochondria were observed with the different kinds of cells.

Effect of the Protonophore, CCCP. To establish that the mitochondrial accumulation of DiOC₇(3) in intact plant cells was related to mitochondrial membrane potential, CCCP was tested for its effect on dye-mitochondria interaction. This protonophore is known to dissipate mitochondrial membrane potential. It was applied at 1 μM, the lowest concentration that consistently stopped cytoplasmic streaming in barley coleoptile epidermal cells. Cells were prestained with DiOC₇(3). Exposure to CCCP (Fig. 6A) led to the rapid release of the fluorescent dye from mitochondria giving a diffuse, low level cytoplasmic fluorescence with no mitochondrial fluorescence observable. The process took place within 1 to 5 min of exposure to CCCP (Fig. 6A). Fluorescence intensity decreased to about 30% of initial values within 5 min for onion and barley cells and to about 45% within 10 min for maize cells (Fig. 6A). Immediately after the addition of CCCP, fluorescence intensity peaked transiently (Fig. 6A). This resembled transient peaks obtained with haustorial mitochondria (4) and probably represented a period in which dye was released from mitochondria but before the dye diffused out of the cell.

Optimal Concentration for DiOC₇(3). The optimal concentration of DiOC₇(3) as an indicator of mitochondrial potential was determined by monitoring the change in fluorescence intensity induced by 1 μM CCCP in the presence of various concentrations of the dye. For all three plant species tested, the optimal concentration was 2 μM. This was the concentration at which the largest change was observed from before addition of CCCP to 15 min later (Table II). In these experiments the residual background fluorescence was somewhat more than in the experiments of Figure 6, because tissues were incubated with dye until CCCP was applied without an intervening hour of incubation on water.

Effects of Cytokinins on Mitochondrial Fluorescence Intensity. Enhancement of mitochondrial fluorescence by kinetin was initially observed qualitatively in barley epidermal cells which had been prepared from coleoptiles, treated with kinetin at 0.1 mM for 1 h, then given DiOC₇(3) 2 μM for 1 h, and finally incubated on water for another hour before observation of fluorescence. The fluorescence of the mitochondria was much brighter in kinetin-treated than in untreated tissues. Furthermore, cytoplasmic streaming seemed to increase after kinetin treatment as observed by movement of fluorescent mitochondria.

Only slight increase in fluorescence intensity in barley epidermis was observed with kinetin at 10 μM, and no effects were observed at 1 μM (data not shown). Zeatin, another cytokinin, did not show any effects on fluorescence of barley mitochondria at 100, 10, or 1 μM (qualitative observations).

To demonstrate the effects of kinetin quantitatively, fluorescence was monitored photometrically before and after kinetin was applied. Cells of onion epidermis, barley epidermis, and maize tissue culture callus were prestained with DiOC₇(3) and the background was destained as usual. Fluorescence intensity was measured twice in a period of 10 min. Subsequently, the cells were exposed to kinetin solutions. The response to kinetin was rapid. Fluorescence intensity started to increase at about 5 min, reached a maximum in about 20 min, and remained stable for the next 40 min (Fig. 6B). The effects were greatest with onion epidermal cells, in which there was an increase of 35% in fluorescence intensity, smallest with maize cells with 18%, and intermediate with barley epidermal cells with 28% (Fig. 6B).

To confirm that the kinetin-induced increase in fluorescence intensity in mitochondria was a consequence of a change in membrane potential, CCCP was applied 60 min after kinetin treatment. As before, fluorescence peaked temporarily after the addition of CCCP and then rapidly declined (Fig. 6B). Fluores-

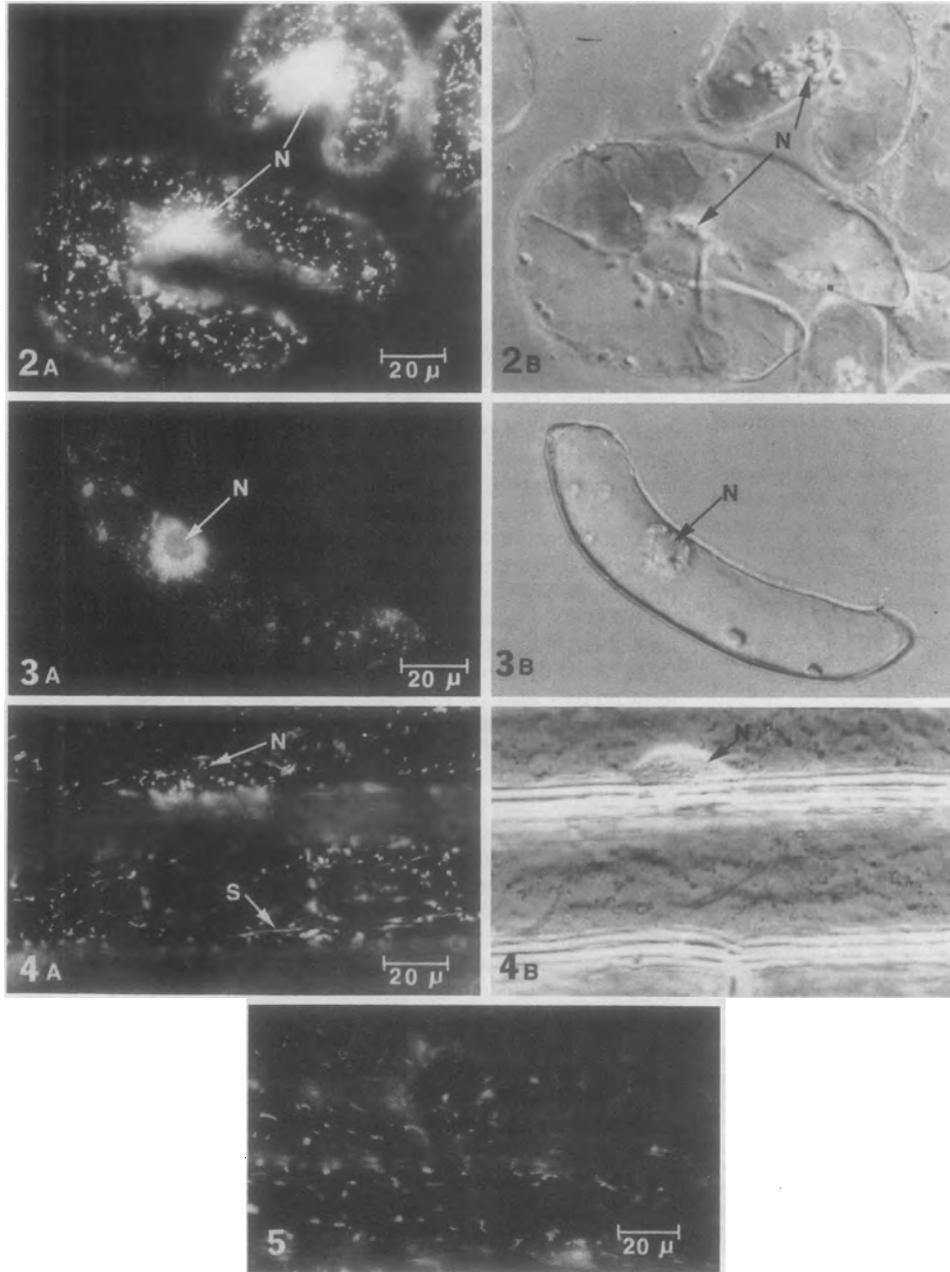


FIG. 2. Maize callus cells stained with DiOC₇(3). A, Epifluorescence microscopy showing numerous mitochondria. Bright fluorescence around nuclei (N) was mostly from surrounding mitochondria (see text). B, Differential interference contrast microscopy of the same field. Scale bar, 20 μ m.

FIG. 3. Maize callus cell stained with DiOC₇(3). A, Epifluorescence microscopy showing numerous bright mitochondria. Bright fluorescence around nucleus (N) was mostly from surrounding mitochondria. B, Transmitted light microscopy of the same field. Nucleus (N) is prominent. Scale bar, 20 μ m.

FIG. 4. Onion cells stained with DiOC₇(3). A, Epifluorescence microscopy showing numerous bright mitochondria which tended to be spherical. Streaks (S) were from mitochondria which moved during photographic exposure. The nucleus (N) was not stained. Mitochondria around the nucleus (N) were not as abundant as in maize cells (Figs. 2 and 3). B, Phase contrast microscopy of the same field. Scale bar, 20 μ m.

FIG. 5. Barley coleoptile epidermal cells stained with DiOC₇(3). Epifluorescence microscopy showing numerous mitochondria. Cell walls (out of focus) show diffuse fluorescence. Scale bar, 20 μ m.

cence intensity of control cells was measured only with barley epidermal cells but the residual fluorescence after CCCP treatment was as low or lower for kinetin-treated cells as for the untreated barley cells in this experiment (Fig. 6B) or for untreated maize and onion cells measured earlier (Fig. 6A).

DISCUSSION

The structures which stained prominently in these experiments with DiOC₇(3) in living plant cells were mitochondria. This was clearly indicated by the morphology, behavior, and response to protonophores of the organelles. They showed a dynamic pleomorphism ranging from filaments to spheres and an ability to shift rapidly from filaments to spheres when cells were disturbed, all characteristic of mitochondria (11). The excellent cytological visualization of mitochondria with cyanines here in living cells of barley, maize, and onion has also been shown for mitochondria within animal cells (5, 13), within cells of carrot and tobacco

(18), protoplasts of alfalfa (26), and in haustoria of the fungus *Erysiphe graminis* (4).

The mitochondria rapidly lost fluorescence upon treatment of cells with CCCP, a protonophore known to depolarize mitochondria. This indicates that accumulation of dye was dependent on the membrane potential that exists across the inner mitochondrial membrane, in agreement with chemiosmotic theory (22) and with other studies with potentiometric dyes in which fluorescence of cyanines in mitochondria was diminished or eliminated by respiratory inhibitors such as cyanide, azide, or rotenone (13).

Other evidence that fluorescence intensity of cyanine relates to mitochondrial membrane potential includes: (a) yeast mutants that possessed mitochondria but were respiration-deficient had no change in fluorescence upon addition of protonophores (15); (b) fluorescence intensity of cyanines has correlated positively with membrane potentials in cells as measured by microelec-

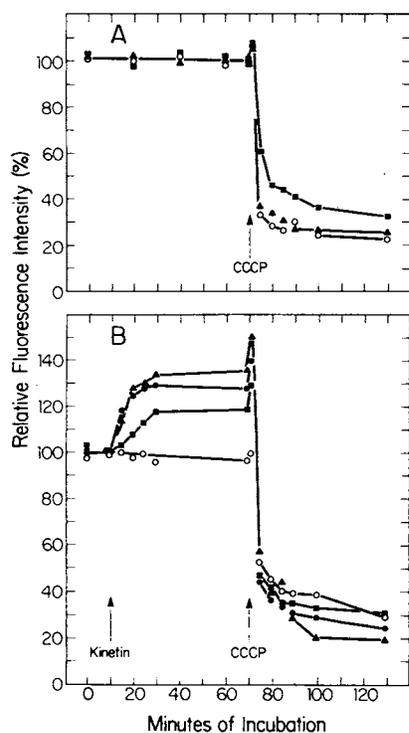


FIG. 6. Mitochondrial fluorescence change in response to CCCP and kinetin. Cells were stained for 1 h with DiOC₇(3) and incubated 1 h in water before measurements were started. A, With 1 μ M CCCP added as shown, without kinetin. Barley (O), maize (■), onion (▲). B, With kinetin 0.1 mM added before 1 μ M CCCP as shown. Barley with kinetin (●), barley (control) without kinetin (O) maize with kinetin (■), onion with kinetin (▲).

Table II. Changes in DiOC₇(3) Fluorescence with CCCP 1 μ M as a Function of Dye Concentration in Cells of Onion, Barley, and Maize

Concentration of DiOC ₇ (3) μ M	F/F ₀ ^a		
	Onion	Barley	Maize
20	0.60	0.81	0.82
10	0.53	0.78	0.80
2	0.42	0.45	0.62
1	0.50	0.56	0.68
0.1	0.57	0.61	0.79

^a Fluorescence intensity 15 min after addition of CCCP/fluorescence intensity before addition of 1 μ M CCCP. Tissues were incubated with dye solution until CCCP solution was applied.

trodes (9, 10, 27, 28), or as estimated from distribution of chloride at Donnan equilibrium (1, 6); and (c) fluorescence increases or decreases with treatments that hyper- or hypopolarize cells (9, 10, 28).

Although mitochondria gave much brighter fluorescence than did other structures in our experiments with DiOC₇(3), faint fluorescence was frequently seen in cell wall regions, in cytoplasm, and in intracellular strands extending in the long dimension of cells. We did not determine if any of the fluorescence in cell wall regions was from the plasmalemma. Fluorescence of intracellular strands was reported by Matzke and Matzke (18) and interpreted to be due to elements of cytoskeleton. In line with this, we found the strands to be located within the cytoplasm and often to coincide with paths of mitochondrial movement.

Matzke and Matzke (18) reported that nuclear membranes fluoresce with the cyanine DiOC₆(3). They showed intense fluo-

rescence associated with the nucleus of a carrot suspension cell, much as we found with maize callus cells (Figs. 2, 3). However, in focusing up and down on such nuclei, we judged that most of the fluorescence was from mitochondria clustered around the nuclei. Many nuclei of onion (Fig. 4) and barley (Fig. 5) epidermal cells were not fluorescent.

The results of Matzke and Matzke who used DiOC₆(3) were much like ours with DiOC₇(3) which has one carbon more in its alkyl side chains. We did not test DiOC₆(3), but found that DiOC₇(3) gave better mitochondrial fluorescence than did DiOC₅(3) or cyanines with shorter side chains. Apparently, both DiOC₆(3) and DiOC₇(3) can be recommended as potentiometric dyes for plant mitochondria. However, Reich *et al.* (26) recently reported that DiOC₂(3) with ethyl side chains stained mitochondria of isolated alfalfa protoplasts.

Whereas we obtained no mitochondrial stain with rhodamine 123, a dye commonly used for mitochondria of animal cells (3, 7), this dye has been reported to stain plant mitochondria in cells (18) and in isolated protoplasts (26). In cells, mitochondrial fluorescence was coupled with intense background fluorescence which interfered with observation of mitochondria.

We found that the fluorescence intensity of mitochondria stained with DiOC₇(3) could be measured photometrically within living plant cells, so that changes in response to experimental treatment could be monitored quantitatively. This was facilitated by the remarkable uniformity of fluorescence intensity of groups of mitochondria as detected in the 314 μ m² aperture of the photometer system. To avoid effects of photodestruction, we moved from site to site within cells and from cell to cell, yet generally found agreement in successive measurements (as in the data of Fig. 6). This agreed with the qualitative observation that fluorescence intensity of all mitochondria within given cells appeared uniform and that fluorescence of mitochondria in different cells within a mount was also uniform.

High concentrations of kinetin (10–100 μ M) increased the fluorescence intensity of mitochondria stained with DiOC₇(3). The response occurred within 5 to 10 minutes and could be attributed to an increase in membrane potential since the protonophore CCCP rapidly dissipated the fluorescence to residual background levels (Fig. 6B). Zeatin had no effect on mitochondrial fluorescence.

Our results do not bear directly on the possible reasons why kinetin increased mitochondrial membrane potential. Miller and co-workers (19–21, 23) found that high concentrations of cytokinin inhibited respiration in both intact plant cells and isolated mitochondria. Their studies with respiratory inhibitors on isolated mitochondria implicated the alternate (cyanide-resistant) pathway and suggested that cytokinins "... influence a function at a point between internal NADH dehydrogenase and cytochrome *b* of the mitochondrial electron transport system ...". (20). This would tend to reduce mitochondrial membrane potential instead of increasing it as suggested by our results with cyanine dyes. Thus, the relation between inhibition of respiration and enhancement of cyanine fluorescence needs investigation. Whatever the mechanisms involved, fluorescent cyanine dyes offer a convenient way to monitor rapid responses to cytokinins.

Acknowledgments—We thank Ed. Stadelmann, Department of Horticulture and Landscape Architecture, University of Minnesota, who supplied onion bulbs for this study; S. Sung, department of Agronomy and Plant Genetics, University of Minnesota, who provided the maize tissue cultures; and Colleen Curran, who provided photoprocessing and layout assistance.

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