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Accumulation of potentiometric and other dyes in haustoria of *Erysiphe graminis* in living host cells†

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Twenty-five vital fluorescent dyes were tested for accumulation in haustoria of *Erysiphe graminis* (DC) Merat f. sp. *hordei* Em. Marchal when applied to coleoptile epidermal host cells of *Hordeum vulgare* L. Eight of the dyes gave fluorescence to structures judged to be haustorial mitochondria. The potentiometric cyanine dyes, $DiOC_4(3)$ and $DiOC_7(3)$ gave best differentiation between the putative mitochondria and other structures. As measured photometrically, fluorescence of the organelles diminished within a few minutes after DNP (1×10^{-4} M), CCCP (1×10^{-6} M), or KCN (1×10^{-3} M) was applied to host tissue, indicating that fluorescence depended on the membrane potential of the organelles.

Many of the tested dyes, including several cyanines, accumulated in haustorial cytoplasm (excluding mitochondria or vacuoles), but the uptake of most of these dyes was not affected by DNP or CCCP, indicating that the dyes moved passively into haustoria. However the uptake of the cyanines $DiOC_5(3)$ and $DiSC_2(3)$ was slightly reduced by CCCP suggesting that a component of uptake of these dyes depended on the potential of the plasmalemma or extrahaustorial membrane of the haustorium.

The cyanine potentiometric dyes provide a way to monitor electrical potential across mitochondrial and other membranes which, in turn, may relate to functional competence of haustoria.

INTRODUCTION

Many vital dyes have been shown to enter living cells of higher plants and fungi, usually by passive diffusion [*7, 24*]. These dyes often accumulate within cells by being bound to intracellular components or by becoming ionized at the pH levels prevailing within the vacuole and therefore unable to exit through the tonoplast. In recent years, a number of potentiometric dyes have been developed which partition across biological

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membranes in proportion to the electrical potential differences across the membranes [*3, 8, 9, 23, 25, 26*]. Of special interest for use in host–parasite investigation are the "slow", positively charged, potentiometric fluorescent dyes that enter cells, mitochondria, or lipid vesicles in proportion to the inside, negative membrane potential of the cell or organelle.

We speculated that the slow potentiometric dyes might be useful for investigating uptake processes by haustoria of *Erysiphe graminis* (DC) Merat f. sp. *hordei* Em. Marchal from host cells of *Hordeum vulgare* L. We screened a number of such dyes for possible active uptake by haustoria from living host cells. For comparison we also screened other positively charged vital dyes as well as some negatively charged potentiometric dyes. Several of the positively charged potentiometric dyes gave fluorescence to structures judged to be haustorial mitochondria. We present here the results of these screening trials and evidence that fluorescence of the organelles is dependent on their membrane potential.

MATERIALS AND METHODS

Host and parasite

Two compatible host–parasite combinations were used: "Proctor" barley (*Hordeum vulgare*) with a culture of race A1-1, *Erysiphe graminis* f. sp. *hordei;* and "Algerian/4* (F-14) Man. S" ("Alg S") barley with culture CR3 of race 3. To obtain a monolayer of host epidermal tissue, coleoptiles from 7-day-old barley plants were partially dissected and mounted as described by Mendgen & Dressler [*19*] except that the adaxial surface of the monolayer was positioned uppermost; the abaxial surface was placed on 0·01 M $CaCl_2$ or $Ca(NO_3)_2$. The dissected coleoptile piece was held on a glass slide with two cover slips, one covering the apex, the other covering the base, leaving a 1·0–1·5 cm segment of the piece uncovered for inoculation and observation. The mounted coleoptile pieces were inoculated in a settling tower with spores of *E. graminis* so that approximately one-third of the epidermal cells 2 days later each hosted a mildew colony. The inoculated specimens were incubated over 20% glycerol (80% RH) in transparent plastic boxes at 18 °C with, daily, 16 h of 4000 lux light, or over saturated $ZnSO_4$ (90% RH) in the dark. Spores for inoculum were grown as described by Bushnell [*4*]. Inoculated specimens usually were used 2–3 days after inoculation when there was a spore, an appressorium, a primary haustorium and one or more hyphae at each infection site.

Solutions

The dyes, their sources, and the concentrations used are listed in Table 1. Each dye was dissolved by placing it in 0·5–1·0 ml of 95% ethanol, then adding potassium phosphate buffer, 0·01 M, to give a dye concentration of 3×10^{-5} M. The pH of the buffer used for each dye is listed in Table 1. Most dyes dissolved in ethanol, but some did not dissolve until buffer was added. The 3×10^{-5} M solution was either used directly or diluted as indicated in Table 1. The final concentration of ethanol was adjusted to 0·5% except for most experiments with $DiOC_4(3)$ and $DiIC_4(3)$ in which 0·02% ethanol was used.

Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Sigma, St. Louis) was dissolved in 100% ethanol and diluted with buffer (pH 6·2 or 6·4) to 1×10^{-6} M with a final ethanol concentration of 0·02%. 2,4-Dinitrophenol (DNP) (Sigma, St. Louis) was dissolved in warm buffer (pH 6·2 or 6·4) and diluted to 1×10^{-4} M. Potassium cyanide was dissolved in buffer (pH 6·2) at room temperature and used at 1×10^{-3} M.

TABLE 1

Fluorescent dyes tested for uptake by haustoria of Erysiphe graminis *in living host cells: type, charge, source, and filter set used for each*

Type	Dye	Charge of dye	pH of buffer	Source[a]	Filter set for epifluorescence[b]	Dye concentration (M)
Cationic, cyanine[c]	$DiIC_1(5)$	+	6·2	A	A	3×10^{-5}
	$DiIC_2(3)$	+	6·4	B	A	1×10^{-6}
	$DiIC_4(3)$	+	6·2–6·4	B	A	1×10^{-6}
	$DiOC_1(3)$	+	6·4	B	B	1×10^{-6}
	$DiOC_2(3)$	+	6·4	B	B	1×10^{-6}
	$DiOC_3(3)$	+	6·4	B	B	1×10^{-6}
	$DiOC_4(3)$	+	6·2–6·4	B	B,C	1×10^{-6}
	$DiOC_5(3)$	+	6·4	B	B	1×10^{-6}
	$DiOC_7(3)$	+	6·4	B	B	2×10^{-6}
	$DiSC_2(3)$	+	6·4	B	B	1×10^{-6}
	$DiSC_3(3)$	+	6·4	B	B	1×10^{-6}
Cationic, other than cyanine	DASPMI[d]	+	6·4	B	B	$0{\cdot}1–1{\cdot}0 \times 10^{-6}$
	4-Di-1-ASP[e]	+	6·2	B	A,B	1×10^{-6}
	Safranin 0	+	6·2	C	D	3×10^{-5}
	Acridine orange	+[f]	8·0	D	B,E	3×10^{-5}
	Nile blue	+	6·2	E	A,B,D	3×10^{-5}
	Rhodamine B	+	6·2	C	A,D	3×10^{-5}
	Rhodamine 123	+	6·4	B	B	$1 \times 10^{-3}–1 \times 10^{-5}$
Anionic, acidic	Sodium fluorescein	−[f]	4·8	D	B,E	3×10^{-5}
Anionic, oxonol	$DiBAC_4(5)$[g]	−	6·2	A	A	3×10^{-5}
	$DiSBAC_2(3)$[h]	−	6·2	B	A	3×10^{-5}
	Oxonol V	−	6·2	A	A	3×10^{-5}
	Oxonol VI	−	6·2	B	A	3×10^{-5}
	Oxonol X	−	6·2	B	A	3×10^{-5}
	Merocyanine 540	−	6·2	A,B	A	3×10^{-5}

[a]Sources for dyes: A, Prof. P. Läuger and H. J. Apell, Faculty of Biology, University of Konstanz; B, Molecular Probes, Eugene; C, Sigma, St. Louis; D, Serva, Heidelberg; E, Merck, Darmstadt.

[b]Filter sets: A, Zeiss green, 510–560; B, Zeiss blue, 450–490; C, Leitz blue, I_2; D, Leitz green, N; E, Leitz violet-blue, H_2.

[c]Designations for cyanines follow Sims *et al.* [*23*].

[d]DASPMI = 2-Di-1-ASP = 2-(4-dimethylaminostyryl)-*N*-methyl pyridinium iodide (Molecular Probes, D-308).

[e]4-Di-1-ASP = 4-(4-diethylaminostyryl)-*N*-methyl pyridinium iodide (Molecular Probes, D-288).

[f]Molecule mostly unionized at pH used.

[g]$DiBAC_4(5)$ = bis-[1,3-dibutyl barbituric acid-(5)]-pentamethineoxonol.

[h]$DiSBAC_2(3)$ = bis-(1,3-diethylthiobarbiturate) trimethineoxonol (Molecular Probes, B-413).

Solutions of dyes or other substances were placed beneath coleoptile pieces and cover slips with a Pasteur pipette after first removing the incubation medium ($CaCl_2$ or $Ca(NO_3)_2$) and then washing the coleoptile piece with the solution to be used. The incubation medium was removed by sucking it out by pipette, holding the tip of the pipette at the edge of the tissue and an edge of each cover slip. The required solution was then injected and removed, to wash the tissue and then fresh solution was applied.

Microscopy and photometry

Tissues were observed with Zeiss incident light fluorescence equipment mounted on either a Zeiss Standard microscope or a Zeiss Photo microscope, or with Leitz incident light equipment mounted on a Leitz Ortholux II microscope. Incident excitation was provided by 100 W (DC) or 50 W (AC) high pressure mercury lamps (Osram). The Zeiss microscopes were equipped with phase and differential interference contrast microscopy for observation by transmitted light. Fluorescence was usually observed with 16 × (N.A. 0·35) or 40 × (N.A. 0·65–0·75) dry objective lenses. The incident light filter sets used with each dye are listed in Table 1. For most dyes, excitation light was blue or green. Green excitation produced no autofluorescence in host or parasite; blue excitation produced moderate yellow autofluorescence in ungerminated or dead spores and in degenerate appressoria as well as faint yellow autofluorescence of host cell walls. Violet-blue excitation, used only with acridine orange and sodium fluorescein, revealed disease-induced autofluorescence in host walls near infection sites as others have reported [*10, 14, 18*].

Fluorescence of $DiOC_4(3)$ and nile blue was measured quantitatively with the Leitz Ortholux II microscope equipped with a Leitz MPV 2 photometer using a Leitz 40 × (N.A. 0·75) dry FLUORESCENZ objective lens. To minimize photodestruction during measurements, an automatic shutter system provided only 0·25 s of excitation for each measurement. A timing device gave measurements at 10-s intervals. Four such measurements were averaged for each reported value. The voltage on the photomultiplier of the photometer was usually set to give a maximum reading of 100–120 for typical haustorial or hyphal cells of the type to be measured.

During measurements, the field diaphram of the vertical illuminator was reduced to 8 μm diameter. The measuring diaphragm of the photometer was set to measure the fluorescence of the specimen in a rectangle, 3·1 × 6·2 μm. This rectangle was positioned within the boundaries of the haustorial central body or the hyphal cell to be measured. Positioning was done with transmitted light microscopy. For each set of four measurements taken in a haustorium or hypha, a paired set was taken with the aperture positioned on the host vacuole to obtain values for background fluorescence. For data on the time course of dye uptake (Figs 3 and 8) the background values are presented; values are given as percentage of the maximum value obtained in the series. For effects of CCCP, DNP and KCN and controls (Figs 4–7), background values were subtracted from values for haustoria; the resulting differences are shown as percentage of haustorial fluorescence at the last reading before test solutions were added.

Staining and observation schedules

With all dyes listed in Table 1, specimens were observed at 10–15 min intervals for 1 h after dye was placed beneath them. Dye was then removed and the specimens were observed at 15–30 min intervals for another hour. To observe effects of protonophores on

dye uptake by haustoria, specimens were pretreated with DNP (1×10^{-4} M) or CCCP (1×10^{-6} M) for 20 min, then dye solution that contained the same concentration of DNP or CCCP as used for pretreatment was added. The development of fluorescence in haustoria in tissues given DNP or CCCP was compared to that in tissues receiving no DNP or CCCP.

To measure uptake of $DiOC_4(3)$ or nile blue photometrically, fluorescence was measured at 5-min intervals for about 60 min after dye was added to specimens. To measure effects of DNP, CCCP or KCN on fluorescence of haustoria already stained with $DiOC_4(3)$, specimens were treated with dye for 1 h, then washed with water or buffer. About 10 min after the dye was removed, photometric measurements were started and taken at 2-min intervals for 8–10 min. The CCCP, DNP or KCN was added to the specimen and a measurement was taken 30 s thereafter (usually) and at 2–5 min intervals for 30 min.

Photography

Photographs were taken either with the Zeiss Standard microscope with a Zeiss M35 camera back and an MC 63 controller, or the Zeiss Photomicroscope. Objective lenses were those described earlier or a 63 × (N.A. 1·4) oil immersion lens for which tissues were placed under a cover slip. To reduce background fluorescence, dye solutions were removed from beneath the coleoptile tissues before photographs were taken: 2–3 h beforehand for nile blue, and 6–18 h beforehand for $DiOC_4(3)$ and $DiOC_7(3)$. Films and range of exposure times used with the Standard microscope were: Ektachrome 400 and Ilford HP5, 0·5–4·0 s. Films used with the Zeiss Photomicroscope were Ilford XP-1-400 and Agfa vario-XL400, with the ASA control set at 1600 for fluorescence microscopy and at 400 for bright field and differential interference contrast microscopy.

RESULTS AND DISCUSSION

Haustorial mitochondria

The dyes listed in Table 1 were applied to the underside of host epidermal tissues. Eight positively-charged dyes imparted fluorescence in structures judged to be haustorial mitochondria (Table 2), the best for mitochondrial fluorescence being the cyanines $DiOC_7(3)$, $DiSC_2(3)$, $DiOC_5(3)$ and $DiOC_4(3)$. These dyes caused fine, thread-like structures, singly or in filamentous chains, to fluoresce [Figs 1(b), (d), (e), 2(a)]. These could be seen most consistently in the finger-like branches of haustoria [Fig. 1(b), (d), (c)], where they were orientated parallel to the longitudinal axes of the branches. The thread-like structures closely resembled in size and orientation the mitochondria in haustorial fingers as viewed by Dahman & Hobot [*6*] by electron microscopy. Similar thread-like structures were also frequently visible in the central bodies of haustoria [Fig. 1(e)]. Small globular structures [Fig. 1(d)] which may also have been mitochondria were sometimes seen. Elsewhere, cyanine dyes have been shown to stain mitochondria within or from animal cells [*5, 11, 15, 21*] or yeast cells [*12, 13, 20*] and, recently, higher plants [*17, 22*]. The accumulation of dye in haustoria could usually be seen within 10–15 min after dye was applied to tissues, even though background fluorescence from dye solution beneath tissues was bright. Uptake could be detected photometrically above background within 15 min after dye was applied (Fig. 3).

Of the four effective cyanines, $DiOC_7(3)$ was the most specific within haustoria for mitochondria followed by $DiSC_2(3)$, $DiOC_5(3)$ and $DiOC_4(3)$ in decreasing order of

TABLE 2

Fluorescence of mitochondria and hyaloplasm of haustoria of Erysiphe graminis *after treatment of host cells with fluorescent dyes in presence or absence of CCCP (1×10^{-6} M)*

Dye	Fluorescence[a]				Special features
	Mitochondria		Hyaloplasm		
	Without CCCP	With CCCP	Without CCCP	With CCCP	
$DiIC_1(5)$	–	×	–	–	Cytoplasm stained light blue with dark blue inclusions (all non-fluorescent)
$DiIC_2(3)$	–	–	++	++	
$DiIC_4(3)$	++	–	++	++	Globular bodies consistently fluoresced, with or without CCCP
$DiOC_1(3)$	–	×	–	×	
$DiOC_2(3)$	–	(Varied)	+	++	Cytoplasmic fluorescence sometimes enhanced by CCCP
$DiOC_3(3)$	–	×	–	×	Globular bodies fluoresced
$DiOC_4(3)$	++	–	+	+	Globular bodies sometimes fluoresce; faint cytoplasmic fluorescence with CCCP
$DiOC_5(3)$	++	–	++	+	
$DiOC_7(3)$	+++	+	++	++	
$DiSC_2(3)$	+	–	++	+	Rapid photodestruction
$DiSC_3(3)$	+	++	+	++	Rapid photodestruction
DASPMI	+	–	+	+,–	Many cells of host and parasite killed; fluorescence varied from cell to cell
4-Di-1-ASP	–	×	+	×	Very weak fluorescence in hyaloplasm
Safranin 0	–	×	–	×	
Acridine orange	–	–	+++	+++	
Nile blue	+	×	+++	+++	Fluorescence of mitochondria seen with filter Set B (Table 1), but rarely
Rhodamine B	–	–	+++	+++	
Rhodamine 123	–	×	–	×	
Sodium fluorescein (Uranin)	–	–	+++	+++	
$DiBAC_4(5)$	–	×	+	++	Rapid photodestruction
$DiSBAC_2(3)$	?	×	++	×	Extremely rapid photodestruction precluded complete evaluation; toxic to host and parasite
Oxonol V	–	×	–	×	
Oxonol VI	–	×	–	×	
Oxonol X	–	×	–	×	
Merocyanine 540	–	×	–	×	

[a]Fluorescence scale: –, absent, rare, or extremely weak; +, weak; ++, moderate; +++, strong; ×, no trial. Scale for mitochondria based on fluorescence of elongate, filamentous structures.

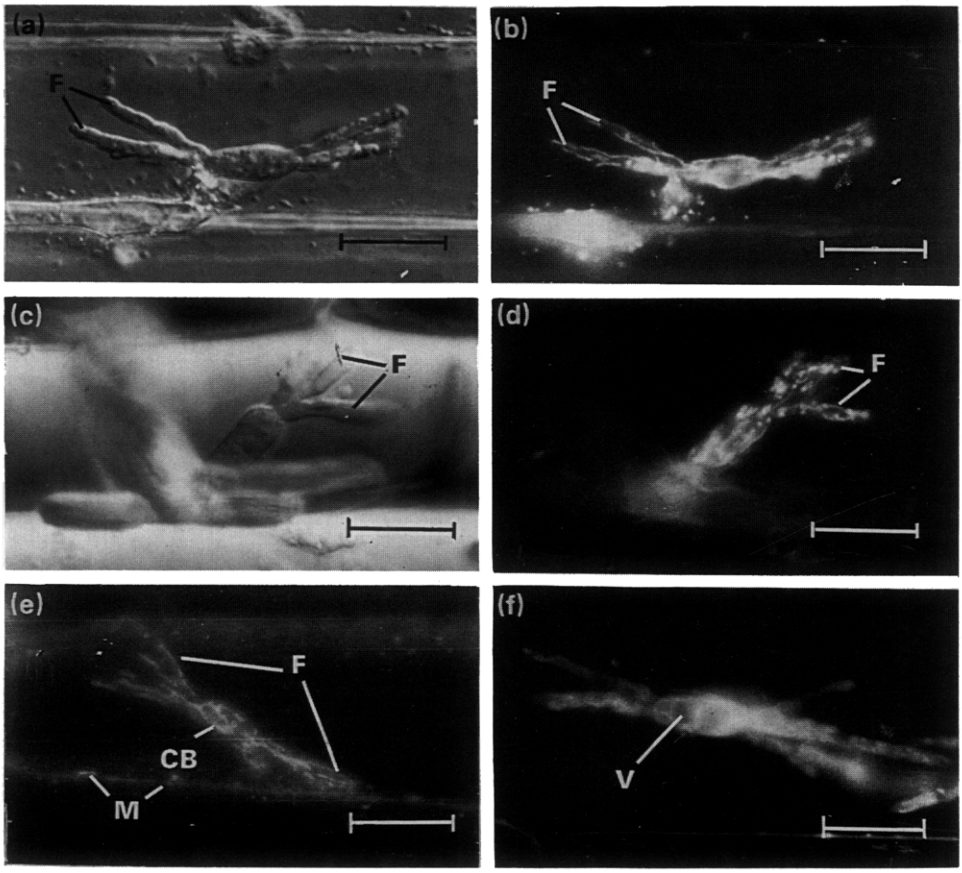

FIG. 1. Haustoria of *Erysiphe graminis* f. sp. *hordei* stained with $DiOC_7(3)$ as viewed by differential interference microscopy [(a) and (c)] or incident light fluorescence microscopy [(b), (d), (f)]. Haustoria are in living epidermal host cells of *Hordeum vulgare*. Scale bars = 20 μm. (a), (b) Brightly fluorescent filamentous mitochondria in focus in haustorial fingers (F). (c), (d), Filamentous and small globular mitochondria fluoresce in haustorial fingers (F). (e) Filamentous mitochondria fluoresce in fingers (F) and central body (CB) of haustorium. Host mitochondria (M) fluoresce along host wall. (f) General fluorescence of haustorial cytoplasm. Such general fluorescence of cytoplasm in stained haustoria resulted from excessive light excitation or application of CCCP or DNP. Unstained vacuole (V) is darker than the surrounding cytoplasm.

specificity. Thus, $DiOC_4(3)$ sometimes stained within haustoria unidentified globular bodies of various sizes most of which were judged to be larger than mitochondria [Fig. 2(b)]; $DiSC_2(3)$ and $DiOC_5(3)$ were intermediate in specificity. All four dyes caused host cell walls to fluoresce weakly and, all conferred a weak general fluorescence to haustorial hyaloplasm (as described later) and to disorganized cytoplasm in dead host cells. Another cyanine, $DiIC_4(3)$, stained haustorial mitochondria (Table 2), but it also consistently stained globular bodies so brightly that mitochondria were difficult to see. DASPMI and nile blue also stained haustorial mitochondria, but only weakly and/or erratically (Table 2). At the concentrations tested, DASPMI was toxic to the parasite as it has been to higher plant cells (Liu, Bushnell & Brambl, unpubl. data).

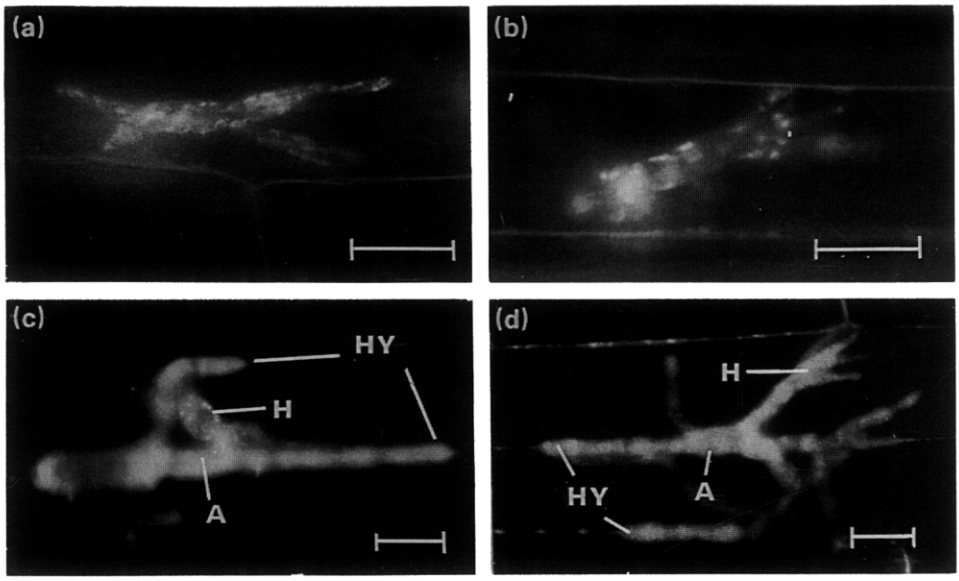

FIG. 2. Haustoria of *Erysiphe graminis* f. sp. *nordei* as viewed by incident light fluorescence microscopy. Haustoria are in living epidermal host cells of *Hordeum vulgare*. Scale bars = 20 μm. (a), (b) Stained with $DiOC_4(3)$. (a) Fluorescent, filamentous mitochondria are distributed throughout a haustorium. (b) Unidentified globular fluorescent organelles, generally larger than mitochondria. (c), (d) Stained with nile blue. Hyphae (HY), Haustoria (H), and appressoria (A) fluoresce. (c) With Blue excitation (Filter set B, Table 1). (d) With green excitation (Filter set A, Table 1).

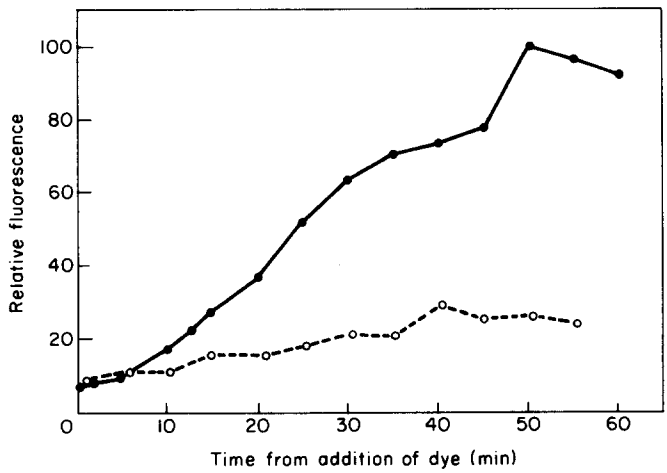

FIG. 3. Uptake of $DiOC_4(3)$ by a primary haustorium of *Erysiphe graminis* f. sp. *hordei*. $DiOC_4(3)$ was applied to the underside of host epidermal cells of *Hordeum vulgare*. Fluorescence was monitored in the central body of a haustorium (●); background (○) was monitored in the host vacuole beside the central body. Relative fluorescence is plotted as percentage of the maximum value obtained.

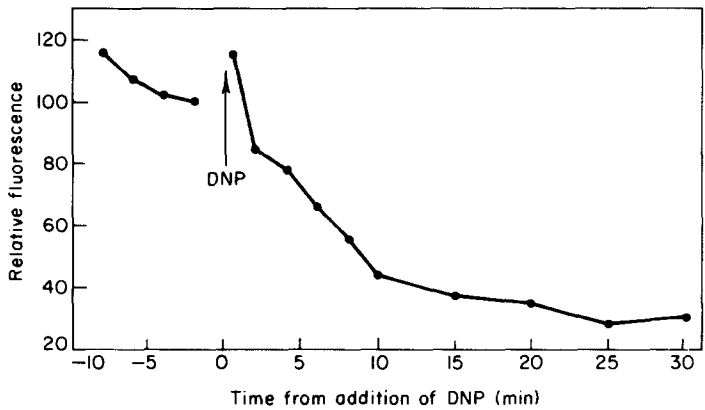

FIG. 4. Effect of DNP (1×10^{-4} M) on fluorescence of primary haustoria of *Erysiphe graminis* f. sp. *hordei*, prestained with $DiOC_4(3)$. Dye was applied to host epidermal tissues for 1 hr and removed before measurements were started. Relative fluorescence is plotted as percentage of the value obtained two minutes before DNP was added. Averages for eight specimens.

Generally the S- and $C(CH_3)_2$-substituted cyanines were less specific for mitochondria than the O-substituted $DiOC_4(3)$ to $DiOC_7(3)$ series. Except for $DiIC_1(5)$, we did not test cyanines with more than three methine carbons [such as $DiOC_4(5)$ or $DiSC_4(5)$] because we lacked the relatively long wavelength incident fluorescence filter sets required for such dyes. Used with suspension cultures of higher plant cells, $DiSC_2(3)$ has given rapid changes in fluorescence in response to treatment with elicitors of phytoalexin production [*16*]. Whether mitochondria were involved was not studied.

Dinitrophenol (1×10^{-4} M) applied with either $DiOC_4(3)$ or $DiOC_7(3)$ prevented haustorial mitochondria from becoming fluorescent; CCCP (1×10^{-6} M) allowed only a weak fluorescence to develop. When either protonophore was added to specimens which had been previously stained, most fluorescence of haustorial mitochondria as viewed directly was lost within a few minutes. This loss could be detected photometrically (Figs. 4 and 5). The decline in fluorescence was preceded by a brief spike of enhanced fluorescence, more prominent and consistent with CCCP (Fig. 5) than with DNP (Fig. 4). The spike occurred whether tissues had been washed in water or buffer before CCCP (in buffer) was added. The spike coincided in time with temporary bright fluorescence throughout the haustorial cytoplasm, as if dye had left mitochondria and imparted a general fluorescence to haustorial cytoplasm [Fig. 1(f)]. This fluorescence began to diminish at once so that haustoria appeared dim by 4 min and were barely visible 8 min after CCCP was applied. As measured quantitatively 10–15 min after DNP or CCCP was applied, fluorescence intensity was 30–40% of that at the time of application (Figs. 4 and 5), mostly from background fluorescence. By this time, DNP-treated haustoria were extremely dim and difficult to see; CCCP-treated haustoria had dimly fluorescent cytoplasm which contrasted with nonfluorescent vacuoles within the haustoria.

When either CCCP or DNP was removed after 1 h, fluorescence of haustorial mitochondria was restored within the next 3 h. Apparently, enough dye remained in the tissue to reaccumulate in mitochondria. Some of the unidentified globular bodies stained with $DiOC_4(3)$ in haustoria tended to lose fluorescence with DNP or CCCP, suggesting that some of the bodies were mitochondria.

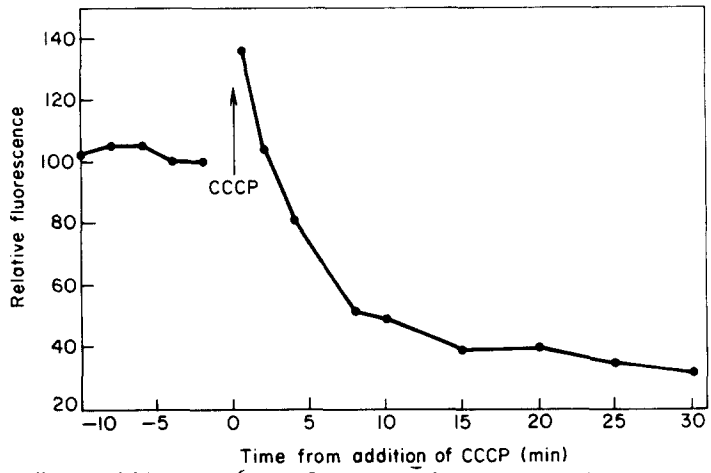

FIG. 5. Effect of CCCP (1×10^{-6} M) on fluorescence of primary haustoria of *Erysiphe graminis* f. sp. *hordei*, prestained with $DiOC_4(3)$. Conventions as for Fig. 4. Averages for five specimens.

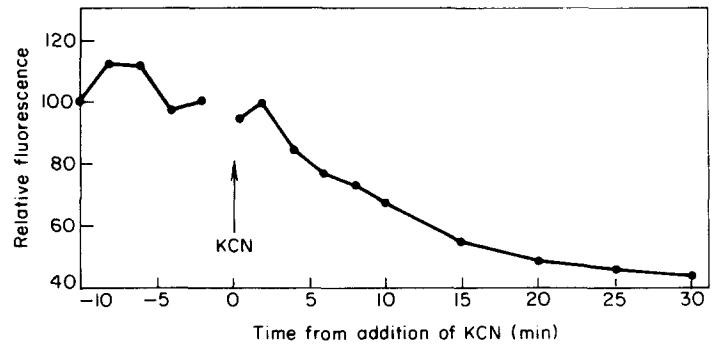

FIG. 6. Effect of KCN (1×10^{-3} M) on fluorescence of primary haustoria of *Erysiphe graminis* f. sp. *hordei*, prestained with $DiOC_4(3)$. Conventions as for Fig. 4. Averages for three specimens.

Potassium cyanide (1×10^{-3} M) also reduced fluorescence of haustorial mitochondria (Fig. 6) but usually without a spike of enhanced fluorescence. The decline in fluorescence with KCN (Fig. 6) was less than with DNP (Fig. 4) or CCCP (Fig. 5), but more than that which occurred in untreated control tissues (Fig. 7).

The loss of fluorescence from haustorial mitochondria produced by CCCP or DNP supports the hypothesis that the fluorescence depended on the electrical potential across mitochondrial membranes. These protonophores rapidly depolarize membranes by allowing protons to move through them. The slower loss of fluorescence caused by KCN, is consistent with the fact that KCN inhibits electron transport and therefore prevents maintenance of membrane potential, but does not immediately depolarize the membrane. That such fluorescence of mitochondria is related to mitochondrial membrane potential has been demonstrated by many investigators using both animal and yeast mitochondria [*9, 12, 13, 15*]. We doubt that potentials of the haustorial plasmalemma, the extrahaustorial membrane, or the host cell plasmalemma contributed much to the fluorescence of haustoria because the weak to moderate fluorescence seen in the

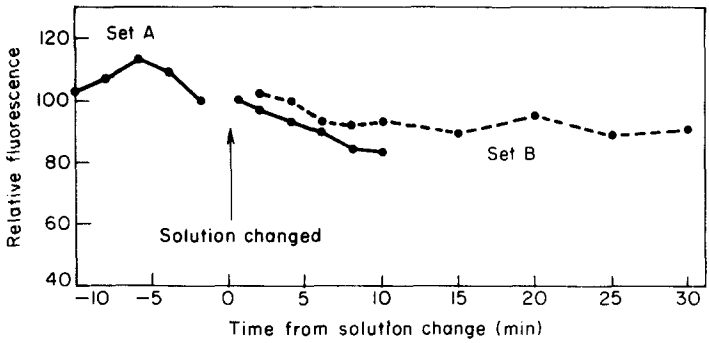

FIG. 7. Relative fluorescence in untreated controls for experiments of Figs 4–6. Specimens were prestained with $DiOC_4(3)$. Conventions as described for Fig. 4 except that tissues were given fresh buffer alone instead of buffer with protonophore or inhibitor when the solution was changed. ●——● Set A, eight specimens; ●– – –●, set B, nine specimens, no data before solution change.

cytoplasm other than in mitochondria usually was not reduced by CCCP or DNP (as described later).

In contrast to $DiOC_4(3)$ or $DiOC_7(3)$, the weak fluorescence of haustorial mitochondria stained with $DiSC_3(3)$ was enhanced if CCCP (1×10^{-6} M) was added at the time dye was applied to host tissues. Fluorescence of haustorial hyaloplasm was also enhanced as described later.

Excessive incident excitation light eliminated the fluorescence of haustorial mitochondria stained with $DiOC_4(3)$ or $DiOC_7(3)$. This effect was partly a result of photodestruction but haustorial cytoplasm temporarily became generally fluorescent [as in Fig. 1(f)] much as happened when CCCP or DNP was added, suggesting that the excitation lowered the ability of mitochondria to hold dye. Care had to be taken to avoid excessive excitation when a series of observations was made. Generally, mitochondrial fluorescence was maintained for 2–3 h when tissues were observed with 25 × dry objective lenses once every 15–20 min, especially if no one area of the tissue was observed for more than a minute at a given time. For a series of frequent visual observations of a single haustorium, we limited each observation to about 5 s. Maintenance of fluorescence for photometric measurements was not a problem because excitation for each measurement was only 0·25 s (total of 1·00 s for the four individual measurements taken at each observation time). Mitochondria in control specimens usually remained bright to the eye throughout a series of measurements, as in Fig. 7. For photography, especially with 40–63 × oil immersion objective lenses which gave intense incident excitation, we had difficulty maintaining haustorial fluorescence for the few seconds required for selecting and focusing the specimen and exposing the film.

Concentrations of $DiOC_4(3)$ at 3×10^{-6} M or higher were toxic to both host and parasite. At 1×10^{-6} to 2×10^{-6} M, no detrimental effects on either host or parasite were observed. $DiOC_4(3)$ or $DiOC_7(3)$ could be applied at 1×10^{-6} M for 1 h, and the haustorial mitrochondria would fluoresce brightly 24–40 h later while hyphae of the fungus grew normally and cytoplasmic streaming continued in host cells. Background fluorescence diminished by 24 h after the dye was applied, so that the contrast between fluorescent mitochondria and the background was improved for photography or other purposes.

Occasionally, a single coleoptile-mount of tissue, or group of mounts had little or no fluorescence of haustorial mitochondria. The reason for this variability was usually unknown, but we suspect that both host and parasite must be in excellent physiological condition to exhibit haustorial fluorescence with cyanine dyes.

The cyanines that were most effective in staining haustorial mitochondria [$DiOC_7(3)$, $DiOC_5(3)$, $DiOC_4(3)$ and $DiSC_2(3)$] also stained host mitochondria [Fig. 1(e) and Liu, Bushnell & Brambl, unpubl. data]. As was the case for haustoria, $DiOC_7(3)$ was the most specific for mitochondria in the host, whereas the three other cyanines imparted fluorescence to other components of host cytoplasm in various amounts. The fluorescence of host mitochondria and other cytoplasmic components tended to reduce contrast between background and fluorescence of haustorial mitochondria. Because it conferred relatively weak fluorescence to host mitochondria, background fluorescence was less with $DiOC_4(3)$ than with the other three dyes.

The dyes $DiOC_4(3)$ and $DiOC_7(3)$ applied to host tissues sometimes appeared first in haustoria, second in attached appressoria and finally, in a few instances, in attached hyphae, especially in hyphal tip cells. However, fluorescence of haustorial organelles was usually brighter and more persistant than was fluorescence of organelles in other fungal structures.

Haustorial cytoplasm

One or more dyes of all the types listed in Table 2 accumulated in haustorial cytoplasm (excluding mitochondria and vacuoles). Thus, the cyanines that stained haustorial mitochondria produced weak to moderate fluorescence of haustorial cytoplasm. As judged visually, the accumulation of dyes in haustorial cytoplasm usually was not impaired by CCCP (Table 2) or DNP (data not shown), which indicates that dye uptake was passive. However the rate of uptake of $DiOC_5(3)$, $DiSC_2(3)$ and DASPMI was reduced slightly by CCCP suggesting that a small component of uptake for these dyes was active. This deserves further investigation as a possible indicator of active uptake by the haustorial plasmalemma or possibly the extrahaustorial membrane. In some instances, movement of potentiometric dyes into cells through the plasmalemma has been demonstrated to be all or partly a function of membrane potential [*1, 2, 13*]. On the other hand, we found the uptake into cytoplasm of $DiOC_2(3)$ and $DiSC_3(3)$, both positively-charged, and $DiBAC_4(5)$, negatively-charged, was enhanced by CCCP, a surprising result since it is unlikely that CCCP opened channels through which these dyes could move.

Haustorial cytoplasm fluoresced more brightly with acridine orange, nile blue, rhodamine B and sodium fluorescein than with other dyes. Uptake of acridine orange was better at pH 8·0 than 6·2; and that of sodium fluorescein better at pH 4·8 than 6·2 (comparative data not shown), in line with the well-known fact that these dyes enter living cells mostly in nonionized form [*24*]. Nile blue gave the highest contrast between haustorium and host and also stained appressoria and hyphae intensely [Figs. 2(c) and (d)]. Nile blue was not toxic to either host or parasite, as brightly fluorescent hyphahaustoria and hyphae could be monitored photometrically (Fig. 8), showing e remained fluorescent overnight and grew normally. Uptake of nile blue by that fluorescence of haustoria increased above background within 10 min after the dye was applied to host tissue; fluorescence of hyphae within 30 min. The progression of fluorescence suggested that dye moved sequentially through the haustorium and the appressorium into

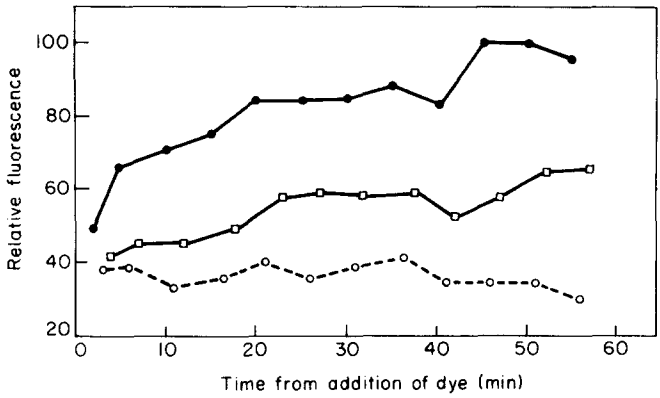

FIG. 8. Uptake of nile blue by a secondary haustorium (●) and a hypha (□) of *Erysiphe graminis* f. sp. *hordei*. Nile blue was applied to the underside of host epidermal cells of *Hordeum vulgare*. Procedures as described for Fig. 3 except that measurements were also taken in a hyphal cell of the colony attached to the haustorium. O----O, Background.

hyphae. However, in a trial 7 h after inoculation, before haustoria were formed, nile blue accumulated readily in appressoria.

Accumulation of nile blue and other cationic dyes (other than cyanines) in haustoria was not significantly reduced by DNP or CCCP, (Table 2) indicating that uptake was probably a result of passive diffusion of the dyes through cell membranes and subsequent accumulation within the haustoria. Nile blue has been widely used as a vital dye for plant cells in which uptake has been unaffected by KCN or by an N_2 atmosphere [7]. The dye accumulates in vacuoles, cytoplasm and walls. Drawert [7] concluded that the cationic vital dyes cross membranes in undissociated form and accumulate in cytoplasm mainly by binding in undissociated form to lipoidal cytoplasmic constituents. These dyes also tend to bind to flavanoids or tannins in cell sap and to carboxyl groups in cell walls, and also may be trapped by becoming dissociated in acidic vacuoles. Whatever the mechanism, nile blue tended to accumulate and remain more in cells of the parasite than in cells of the host in our trials. We occasionally saw mitochondria stained with nile blue, in line with several reports that nile blue stains plant mitochondria (chondriosomes of Drawert [7]).

The cyanine dyes provide a new tool for study of host–parasite interaction in powdery mildews and other diseases. By indicating membrane potential of mitochondria, these dyes can indicate whether cells are actively maintaining oxidative respiratory activity. They can be used in this way for mitochondria in haustoria, as shown here, and for mitochondria in higher plant cells ([17, 22], Liu, Bushnell & Brambl, unpubl. data). We plan to monitor host and parasite in powdery mildews before and during hypersensitive resistance responses to learn when respiratory activity in mitochondria stops in relation to collapse of host and parasite cells.

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