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Direct Measurement of Acetylesterase in Living Protist Cells[1]

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The fluorogenic acetylesterase (acetic ester hydrolase EC 3.1.1.6.) substrate, fluorescein diacetate, was used to measure enzyme activity in living protist cells. The visual enzyme assay was done by monitoring fluorochromasia by fluorescent microscopy. Quantitative fluorogenic assays were done by measuring the evolved fluorescein in a fluorometer. Of 59 strains of bacteria, 35 were fluorochromatically positive. Eight of the fluorochromatically negative strains were fluorogenically positive. Of 22 strains of slime molds and fungi, all were fluorochromatically positive. Three out of 12 different algae were fluorochromatically positive. Several unidentified protozoa were also fluorochromatically positive. Four out of six protozoa were fluorochromatically positive. Structures of special interest showing acetylesterase activity were: the growing hyphal tips of fungi, the vacuolated areas of yeast and protozoa, newly formed bacterial spores or immature fungal spores, "mesosome-like" bodies in *Bacillus megaterium*, and the cell membrane and nuclear region of green algae. Yeast protoplasts and bacterial protoplasts and spheroplasts were fluorochromatically positive when derived from positive cells and negative when derived from negative cells. There was no correlation between the possession of a capsule and acetylesterase activity. There was no effect on the viability of bacterial cells incubated in the presence of fluorescein diacetate. Paraoxon inhibited bacterial and yeast enzyme at 10^{-5} M. Eserine (10^{-5} M) and Paraoxon (10^{-7} M) inhibited *B. megaterium* enzyme. Sodium acetate at 10^{-2} M did not inhibit bacterial enzyme. The implications of these findings on the location and expression of esterase activity in living cells are discussed.

Esterases have been identified in bacteria (3, 33, 36), in bacterial spores (25, 26, 34), in fungi (5, 22, 23), in algae (27), and in protozoa (1, 11, 19). The enzymes were identified in cell extracts by gel electrophoresis, kinetic assays, and cytochemical localization by light and electron microscopy. The use of a multitude of substrates makes it difficult to compare the enzyme activities of various organisms.

Rotman and Papermaster (29) demonstrated the uptake and enzymatic hydrolysis of fluorescein esters by living animal cells. The released fluorescein was concentrated inside the cell, rendering it visible by fluorescence microscopy. This phenomenon was termed *fluorochromasia*. An eyepiece photometer was used to measure the evolved fluorescein, which followed Michaelis-Menten kinetics. The adjective *fluorogenic* was applied to this process. Fluorescein diacetate (FDA) was found to be the best fluorogenic substrate. The same substrate was used by Roberts and Rosenkrantz (26) to demonstrate acetylesterase (EC 3.1.1.6.) activity in *Bacillus coagulans* cells and spores by a fluorogenic assay, but no report of fluorochromasia was made.

This communication is concerned with the search for fluorochromasia in 100 living protist organisms, as well as the location and quantitation of enzyme activity in those which were positive.

MATERIALS AND METHODS

Organisms tested. Table 1 lists the organisms used. Cultures numbered 1 to 8, 14 to 31, 33 to 38, 40, 41, 43 to 48, 51 to 56, 63, and 64 were from the culture collection of the Department of Bacteriology and Immunology, University of Western Ontario. From this department, numbers 98 and 100 were provided by C. F. Robinow, 57 to 59 by Ruth Ziv, and 95 by H. Bauer. From the Department of Botany, University of Western Ontario, cultures numbered 60, 62, and 65 to 81 were provided by Angela Wellman and 85, 86, 89,

[1] Presented at the 17th annual meeting of the Canadian Society of Microbiologists, Hamilton, Ontario, June 1967.

TABLE 1. *Fluorochromatic and fluorogenic assay for acetylesterase activity with fluorescein diacetate as substrate*

Culture no.	Organism	Growth medium	Cells, hyphae, or plasmodium		Fruiting body (FC)	Mature spores (FC)	Immature spores (FC)
			FC[b]	FG[c, d]			
	Bacteria						
	Pseudomonas aeruginosa	BA[a] or					
1	yellow pigment	NA[a]	+	+			
2	green pigment		+				
3	blue-green pigment		+				
4	*Spirillum serpens* VHL	S & C[a]	+	+			
5	*S. serpens* VHA	S & C	+	+			
6	*S. serpens* MW5	S & C	+	±			
7	*Aerobacter-Klebsiella*	NA	−	−			
8	*Alcaligenes faecalis*	NA	−	−			
9	*Escherichia coli* (laboratory strain)	NA	−	−			
10	*E. coli* (human isolate)	NA	−	−			
11	*E. coli* K -12 Hfr λ+	NA	−	−			
12	*E. coli* B spheroplasts	NA	−	−			
	E. coli spheroplasts	NA	−	±			
13	*E. coli* 600 C F^-	NA	−	−			
14	*E. coli* Lilly	NA	−	±			
15	*E. coli* 0111:B4	NA	+	+			
16	*Erwinia grula*	YG[a]	−	−			
17	*Proteus vulgaris*	BA	+	+			
	P. vulgaris spheroplasts		+	+			
18	*Salmonella typhi*	NA	+	+			
19	*S. paratyphi* B Bryan 1	NA	+	+			
20	*Serratia marcescens*	BA	+	+			
21	*Shigella flexneri* 2_a	NA	−	−			
22	*S. flexneri* I	NA	−	−			
23	*S. sonnei*	NA	+	+			
24	*Neisseria catarrhalis*	BA	+	+			
25	*Micrococcus aureus*	YG	−	−			
26	*M. citreus*	YG	+	+			
27	*M. lysodeikticus*	YG	−	−			
28	*Staphylococcus aureus* (lab strain)	NA	−	−			
29	*S. aureus* PS6	NA	+	+			
30	*S. aureus* PS3B	NA	+	+			
31	*Corynebacterium equi*	BA	+	+			
32	*Diphtheroids*	BA	−	+			
33	*Listeria monocytogenes* L42	NA	+				
43	*L. monocytogenes* L81	NA	+				
35	*L. monocytogenes* L85	NA	+	+			
	L. monocytogenes protoplasts	NA	+	+			
36	*L. monocytogenes* L109	NA	+				
37	*Diplococcus pneumoniae*	BA	+				
38	*Streptococcus* sp. (haemolytic)	BA	+				
39	*S. viridans*	BA	−				
40	*Bacillus cereus*	NA	+	+		−	+
41	*B. cirulans*	or	−	+			
42	*B. coagulans*	PDA[a]		+[e]		+(FG)[e]	
43	*B. megaterium*	PDA	+	+		+	+
	B. megaterium protoplasts	PDA	+	+			
44	*B. mycoides*	PDA	+			−	
45	*B. polymyxa*	PDA	−	+		−	
46	*B. subtilis*	PDA	+			−	
47	*Clostridium tetani*	BY[a]	+	+			+

TABLE 1—*Continued*

Culture no.	Organism	Growth medium	Cells, hyphae, or plasmodium		Fruiting body (FC)	Mature spores (FC)	Immature spores (FC)
			FC[b]	FG[c, d]			
48	*C. welchii*	BY	+	+			+
49	*Streptomyces chrysomallus* S-5	NA					+
50	*S. chrysomallus* S-17	NA	+				
51	*Cytophaga johnsonii* 405	TA[a]	−	+			
52	*Cytophaga* sp. 9D	TA	−	+			
53	*Sorangium* sp. 495	TA	+				
54	*Myxobacter* sp. 4449	BCA[a]	+		+		
55	4497	BCA	+		+		
56	P2	BCA	+		+		
57	*Mycoplasma gallisepticum*	ED[a]	−				
58	*M. laidlawii* A	ED	−				
59	*M. laidlawii* B	ED	−				
	Fungi						
60	*Dictyostelium* sp.	YPSS[a]	+		+	−	
61	*D. discoideum*	YPSS	+[f]				
62	*Physarum* sp.	MA[a]	+		+		
63	*Saccharomyces cerevisiae* 1376	YG	+				
64	*Lipomyces lipofer*	YG	+	+			
	L. lipofer protoplasts		+	+			
65	*Endomyces magnusii*	MA	+				
66	*Absidia spinosa*	MA	+				
67	*Phycomyces blakesleeanus* 847	MA	+		+	−	
68	*P. blakesleeanus* 931	MA	+				
69	*Fusarium* sp. 393	MA	+		±	±	
70	490	MA	+				
71	941	MA	+				
72	*Torulopsis* sp.	MA	+				
73	*Aspergillus fisheri*	CA[a]	+		+	+	
74	*A. niger* 783	MA	+				
75	*A. niger* 848	MA	+		+	−	+
76	*A. nidulans*	MA	+				
77	*Penicillium chrysogenum*	PDA	+				
78	*P. claviforme*	MA	+				
79	*P. duclauxi*	MA	+				
80	*P. notatum*	CZ	+				
81	*P. roqueforti*	MA	+				
	Algae						
82	*Gleocapsa* sp.	PW[a]	−				
83	*Oscillatoria* sp.	PA	−				
84	*Nostoc* sp.	PW	+				
85	*Carteria* sp.	PW	−				
86	*Pandorina morum*	PW	−				
87	*Cladophora* sp.	PW	−				
88	*Volvox* sp.	TU[a]	−				
89	*Hyrodictyon reticulatum*	PW	−				
90	*Spirogyra* sp.	TU	+				
91	*Closterium lanceolatum*	PW	−				
92	*Tribonema aequale*	PW	+				
93	*Vaucheria* sp.	PW	−				

TABLE 1—*Continued*

Culture no.	Organism	Growth medium	Cell, hyphae, or plasmodium		Fruiting body (FC)	Mature spores (FC)	Immature spores (FC)
			FC[b]	FG[c,d]			
	Protozoa						
94	*Euglena gracilis*	W & G[a]	−				
95	*Acanthameba* sp.	K & N[a]	+				
96	*Amoeba proteus*	PW	+				
97	*Paramoecium caudatum*	W & G	+				
98	*Stentor coeruleus*	PW	−				
99	*Tetrahymena pyriformis*		+[f]				
100	Unknown protozoa	PW	+				

[a] Growth media: BA, blood agar; BCA, bacterial cell agar; BY, brewer's yeast broth; CZ, Czapeck's agar; ED, Edward's broth; K & N, Klein and Neff broth; MA, malt agar; NA nutrient agar; PDA potato-dextrose agar; PW, pond water; S & C, spirillum broth plus 1-cystine; TA, tryptone agar; TU, Turtox universal broth; W & G, water plus grain; YG, yeast extract- glucose agar; YPSS, yeast extract-peptone-soluble starch-agar.

[b] FC, fluorochromatic, revealed by fluorescence microscopy (29).

[c] FG, fluorogenic, measured by fluorometry (29). FG+ = greater than 5×10^{-3} acetylesterase units per optical density unit.

[d] Total cells (direct count) per optical density unit; culture no. 1, 7–23, 31, 35, 51, 52 contained 2.2×10^9; culture no. 4–6 contained 3.6×10^8; culture no. 24–30 contained 1.5×10^9; culture no. 40, 41, 43, 45, 47, 48 contained 1.4×10^8; culture no. 64 contained 5.9×10^6.

[e] Roberts and Rosenkrantz (26).

[f] Rotman and Papermaster (29).

91, and 92 were provided by D. McLarty. H. B. Stewart, Department of Biochemistry, provided culture number 64. Cultures 49 and 50 were the gift of E. Roslycky, Canada Department of Agriculture, Research Station, London, Ontario. The clinical laboratory of Victoria Hospital, London, Ontario, provided cultures 9 to 13, 32, and 39. Cultures 82 to 84, 87, 88, 90, 93, 94, 96, and 97 were purchased from Turtox (General Biological Supply House, Chicago, Ill.).

Cultivation and preparation of cells for testing. The culture media for the organisms tested are listed (Table 1). Bacteria were usually grown overnight. Fungal cultures were grown on cellophane-covered agar for 48 to 72 hr. Algal and protozoan cultures were 1 to 2 weeks old.

Cells grown in broth were centrifuged at $6,500 \times g$ and washed in saline (0.14 M NaCl) in the case of most bacteria, in culture water in the case of algae and protozoa, or in the appropriate osmotically regulated buffer in the case of *Mycoplasma* (28), protoplasts, and spheroplasts. The cells were resuspended to a slight turbidity for microscopy or to an optical density (OD) of 0.9 at 680 nm in an 0.5-inch (1.27 cm) test tube (Bausch & Lomb, Inc., Rochester, N.Y.) with a Spectronic 20 spectrophotometer for fluorometry. Total cell counts were done on these suspensions by means of a Petroff-Hauser chamber.

With fungi growing on cellophane-covered agar, a small piece of the cellophane-bearing hyphae was cut out, and the hyphae were floated on a drop of water on a slide.

Cells grown on a solid medium were washed off in 5 ml of saline, centrifuged, and resuspended as above.

Preparation of protoplasts and spheroplasts. *Listeria monocytogenes* strain L85 was converted to protoplasts by the method of Ghosh and Murray (9) without lipase treatment. The protoplasts were washed in a solution containing 0.5 M sucrose, 0.03 M tris-(hydroxymethyl)aminomethane(Tris) buffer (*p*H 6.6), 0.02 M $MgCl_2$, and 0.85% NaCl, and resuspended in the same solution.

B. megaterium were converted to protoplasts by the method of Weibull (37). Lysozyme (1 mg/ml; Nutritional Biochemicals Corp., Cleveland, Ohio) in sucrose (0.3 M)-$MgSO_4$ (0.016 M)-phosphate buffer (0.02 M, *p*H 7.2) was used. The cells were washed and resuspended in the buffer without lysozyme.

Escherichia coli spheroplasts were prepared by a modification of the method of Lederberg (15). One volume of washed cells was added to three volumes of fresh Nutrient Broth (Difco) containing 0.67 M sucrose, 0.015 M $MgSO_4$, and 417 units of penicillin per ml. The mixture was shaken in a 37 C water bath for 60 to 90 min. The cells were centrifuged and resuspended in the sucrose-$MgSO_4$-phosphate buffer without Nutrient Broth or penicillin.

Proteus vulgaris spheroplasts were prepared in the same manner as for *E. coli*, but were incubated for longer periods of time.

Lipomyces lipofer protoplasts were prepared according to the method of Heick and Stewart (12), and were kindly supplied by Dr. Stewart.

Microscopy. On a clean slide, one drop of cell suspension was mixed with one drop of FDA (Mann Research Laboratories, Inc., New York, N.Y.) substrate (10^{-3} M) in 0.14 M NaCl for whole cell preparations, or in sucrose buffer for protoplast, spheroplast, and mycoplasma (28) preparations. A clean coverslip

was dropped over the fluid and the edges were sealed with vaseline.

The preparations were examined with a Zeiss large fluorescence microscope with a phase condenser, equipped with a Nikon 35-mm camera; Kodak plus-X panchromatic film (ASA 125) was used. The fluorescent photomicrographs were taken by using a mercury vapor lamp (HB200) with a no. 2 exciter filter, an open condenser, and a no. 50 barrier filter. The usual exposure was 3 min. Ultraviolet absorption photomicrographs were taken as above except the no. 65 barrier filter was used. A 1-sec exposure was usually used. Phase-contrast photomicrographs were taken by using the same optics but substituting white light and a phase condenser system. The usual exposure was made in subdued light for 10 sec. Those cells which developed a yellow-green fluorescence within 1 hr were considered fluorochromatically positive (FC+). All determinations were done at least in duplicate.

Fluorometry. For each enzyme titration, one tube (Bausch & Lomb Spectronic 20, 0.5-inch test tubes) containing 2.0 ml of substrate solution and one containing 2.0 ml of solvent without FDA were used. To each tube, 0.5 ml of cell suspension was added. The suspension was mixed throughout the experiment on a Vortex mixer, and then read at intervals for at least 15 min on a Turner model 110 Fluorometer. A 110–850 ultraviolet lamp with a 110–811 exciter filter and a 110–816 barrier filter were used to monitor the evolution of fluorescein by enzymatic cleavage of the FDA. When the suspension showed too much activity to be recorded on the fluorometer, the cells were appropriately diluted before being added to the substrate. The instrument was calibrated with a standard purified fluorescein solution. The quantity of fluorescein liberated was read from a standard curve. One acetylesterase enzyme unit equals the release of 1 μmole of fluorescein per min at 22 C. The specific activity (Table 2) equals the number of acetylesterase units per cell. For convenience, a cell suspension was considered fluorogenically positive (FG+, Table 1) when it contained more than 5×10^{-3} acetylesterase units per optical density unit.

Inhibitors. To 1.0 ml of substrate solution, 1.0 ml of inhibitor solution was added. A control tube with water instead of inhibitor was prepared. At zero min, 200 μliters of cell suspension was added, and fluorometry was performed as above.

The final concentrations of inhibitor used were: eserine sulfate (physostigmine; Nutritional Biochemical Corp.), 4.9×10^{-5} M; paraoxon (diethyl-*p*-nitrophenyl phosphate; K & K Laboratories, Inc., Plainview, N.Y.), 4.9×10^{-5} to 4.9×10^{-7} M; and sodium acetate (Fisher Scientific Co. Ltd., Toronto, Ontario), 4.9×10^{-2} to 4.9×10^{-6} M.

Viability test. At zero min, 500 μliters of cell suspension (OD_{680} = 0.9) were added to a tube containing 5.0 ml of saline or substrate in saline. Samples were taken at zero, 30, and 60 min; they were then diluted and plated, and the colonies were counted by standard techniques.

TABLE 2. *Relative acetylesterase activity of various living microorganisms and their spheroplasts or protoplasts*

Organism	Age (hr)	Specific activity (units per cell)
Proteus vulgaris cells	3	0.256×10^{-11}
P. vulgaris spheroplasts		0.426×10^{-11}
Listeria monocytogenes cells	6	12.6×10^{-11}
L. monocytogenes protoplasts		16.9×10^{-11}
Lipomyces lipofer cells	18	21.70×10^{-9}
L. lipofer protoplasts		29.10×10^{-9}
Bacillus megaterium cells	5	10.80×10^{-9}
B. megaterium protoplasts		13.30×10^{-9}

RESULTS

Bacteria. It is difficult to draw any generalization about the ability of various groups of bacteria to take up FDA and hydrolyze it (Table 1). Although most bacteria tested were positive, negative examples arose from both gram-positive and gram-negative groups. Of particular note is the low activity shown by *E. coli* and related microorganisms. No distinction could be made on the basis of oxygen requirements, or the presence or absence of a capsule (as measured by light microscopy when the organisms were suspended with India ink particles). The only group of bacteria without cell walls, the mycoplasma, did not show acetyl esterase activity under the test conditions.

Figure 1 shows the distribution of fluorochromasia among a group of *Diplococcus pneumoniae* cells. The sensitivity of the method is indicated in Fig. 2 in which the fluorescein appears to be concentrated in the area normally occupied by mesosomes in young growing cultures of *B. megaterium*. When the same organisms undergo sporulation, fluorochromasia is occasionally seen in these cells (Fig. 3). Similar results were obtained with other spore-forming bacteria.

It was not possible to photograph stained protoplasts or spheroplasts, owing to the fact that the fluorescein escaped from these cells too rapidly, rendering the background very bright. It was always noted that protoplasts or spheroplasts from fluorochromatically positive cells were always fluorochromatically positive and vice versa.

Fungi. All the slime molds and fungi which have been tested were positive for acetylesterase activity. Because of the larger size of most of these organisms, good photomicrographs were obtained. Fig. 4 shows the appearance of *Dictyostelium* sp. after staining with FDA.

In Fig. 5, the exclusion of the fluorescein from

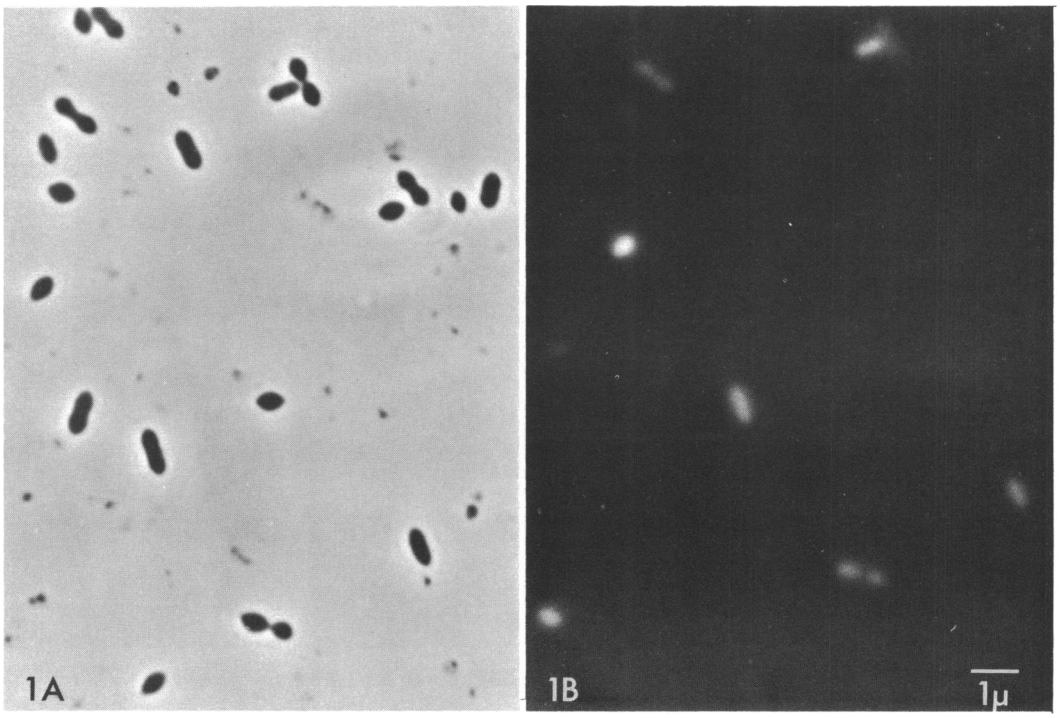

FIG. 1. *Diplococcus pneumoniae. (1a) Phase contrast; (1b) fluorescence; the whole cell fluoresces. Figures 1–11 are paired photographs of the same field. All cells are living and stained for acetylesterase as described in Materials and Methods.*

the lipid droplets within the *Lipomyces lipofer* is clearly visible. The same organisms converted to protoplasts illustrate this effect quite spectacularly (Fig. 6). The vacuoles within the cell were heavily stained but could not be distinguished in photographs.

Endomyces magnusii (Fig. 7) and *Absidia spinoza* (Fig. 8) cells also show localization of the enzyme end product. Of particular importance is the high concentration of fluorescein in the hyphal tips of growing mycelia. All the fungi listed in Table 1 showed fluorescence along the mycelia.

Figure 9 illustrates the appearance of the fruiting body of *Aspergillus niger*. The staining of the younger spores, as well as the internal cytoplasm and foot cell, indicates wide-spread enzyme activity. Many fungi tested showed stained spores and internal particles with fluorescein localization.

Algae. Within the algae, many organisms autofluoresce red or green. This makes the demonstration of acetylesterase activity by this method most difficult. The results in Table 1 represent our best judgement on the acetylesterase activity of these cells. Three out of 12 organisms tested were positive. Figure 10 illustrates identification of the nuclear region as the largest element within the *Spirogyra* sp. cell which concentrates the fluorescein. The cytoplasmic membrane, as well as the membrane surrounding the chloroplast, apparently also has some fluorescein present. The pyrenoids might also contain fluorescein, but this is not easily determined. The chloroplast itself fluoresces a bright red.

In Fig. 11, the chloroplast of *Tribonema aequale* appears to surround the nuclear region which stains brightly with the fluorescein.

Protozoa. Although many of the protozoa also show autofluorescence, it was possible to see fluorochromasia in four out of six species tested. Several unidentified protozoa were also positive. Because of the motility of most of these organisms, it was not possible to slow them down sufficiently to get high-quality fluorescent photomicrographs. It was noted, however, that the fluorescein did concentrate in vacuoles which probably correspond to the food vacuoles seen by others.

Relative esterase activities of various microorganisms. When the specific activities of various microorganisms were calculated, it was found that

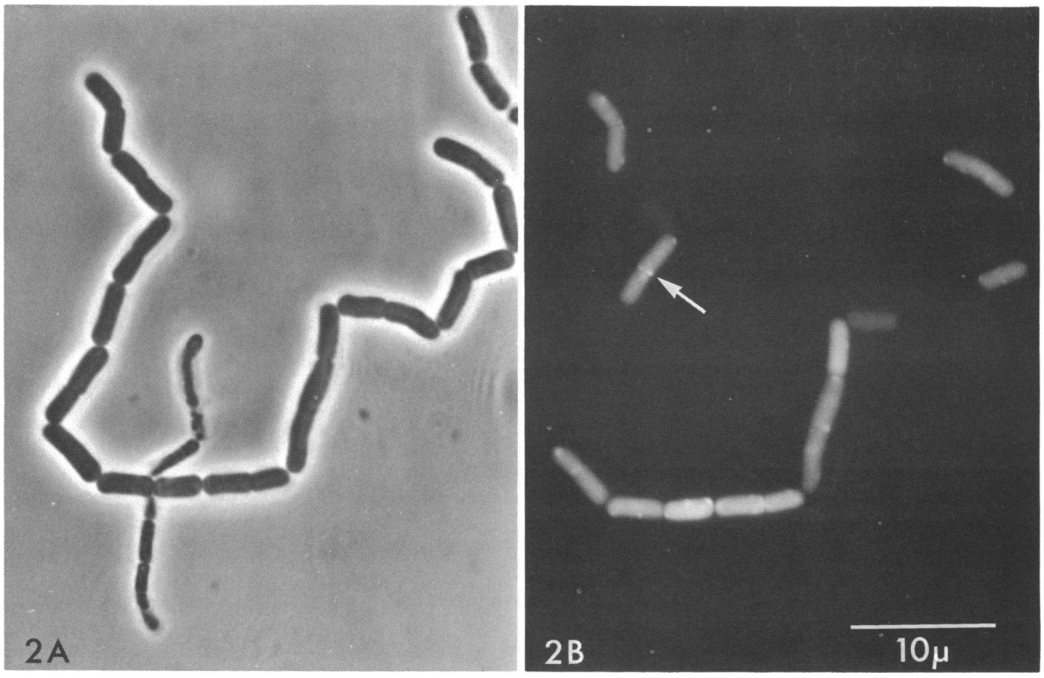

FIG. 2. *Bacillus megaterium. (2a) Phase contrast; (2b) fluorescence. Note the apparent concentration of fluorescein in "mesosomes" (arrow).*

there was a great variability from one microorganism to the other. This variability depended upon the age of the culture, the size of the cell, and whether it was tested as a whole cell or as a protoplast. The activities of *P. vulgaris* cells and spheroplasts, *L. monocytogenes* cells and protoplasts, *L. lipofer* cells and protoplasts, and *B. megaterium* cells and protoplasts are summarized in Table 2. It is obvious that the *L. lipofer* cells and *B. megaterium* cells were very active compared to the others. The protoplast or spheroplast was usually more active than the whole cell.

Effect of potential inhibitors on acetylesterase activity. It was of interest to see whether potential acetylesterase inhibitors would affect the enzyme activity being measured. Table 3 presents the limited data which demonstrates that paraoxon, an inhibitor of arylesterase, exhibits some activity in bacteria and yeast at 5×10^{-5} M. *B. megaterium* was inhibited at 5×10^{-7} M. Eserine, an inhibitor of cholinesterases, slightly inhibited *B. megaterium* at 5×10^{-5} M. Sodium acetate showed no inhibitory activity on *B. megaterium* esterase at 10^{-2} to 10^{-7} M.

Effect of fluorescein diacetate on the viability of microorganisms. Cultures of *B. megaterium*, *Pseudomona aeruginosa*, and *E. coli* B were tested for sensitivity to FDA. In each case, the total number of viable cells did not decrease and showed a slight increase when incubated in FDA over a period of 60 min.

DISCUSSION

There are some inherent problems in classifying acetylesterases in living organisms (2). Rotman and Papermaster (29) used a series of monoesters and diesters of fluorescein on living mamalian cells to demonstrate their uptake and hydrolysis by intact cells. Fluorescein diacetate was found to be the most easily penetrating substrate and had the property of rendering the intact cell bright yellow-green when the evolved fluorescein was retained inside the cell. Roberts and Rosenkrantz (26) referred to a similar enzyme as an acetylesterase. Similar fluorescein and eosin esters have been tested by Guilbault and Kramer (10) as substrates for isolated lipases. Also, a related compound, dichloro-diacetyl-fluorescein has been used as a hydrogen acceptor in the measurement of oxidase activity (14). A further difficulty in the definition of the term "esterase" is that several proteolytic enzymes have esterase activity (6); bacterial enzymes are no exception (18).

Certain difficulties were experienced in photography. The first problem was the motility of

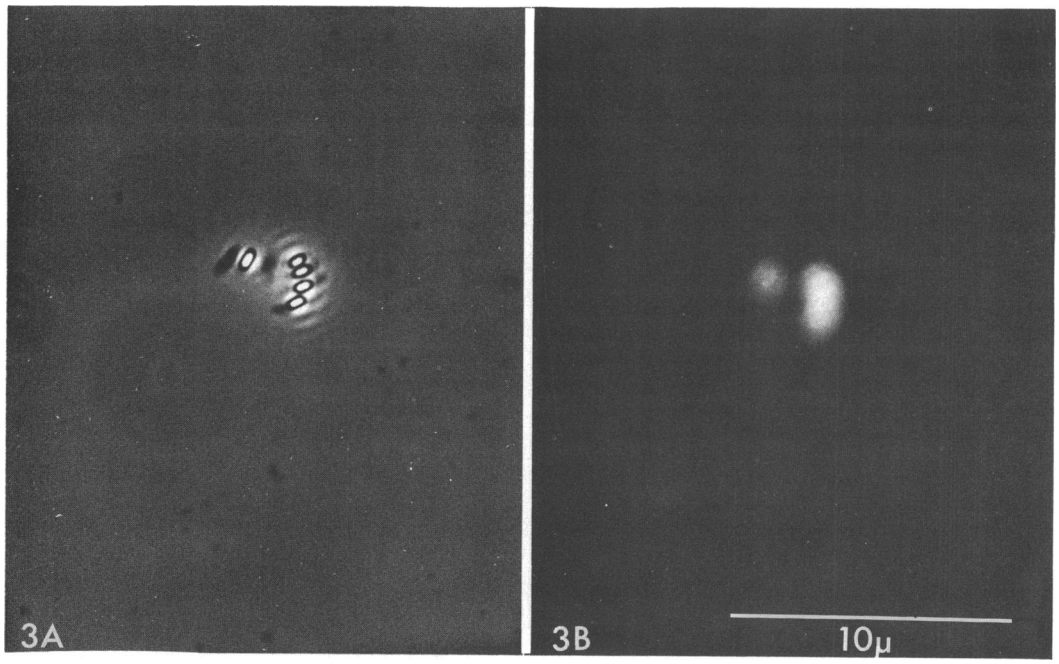

FIG. 3. *Sporulating cells of Bacillus megaterium. (3a) Phase contrast; (3b) fluorescence.*

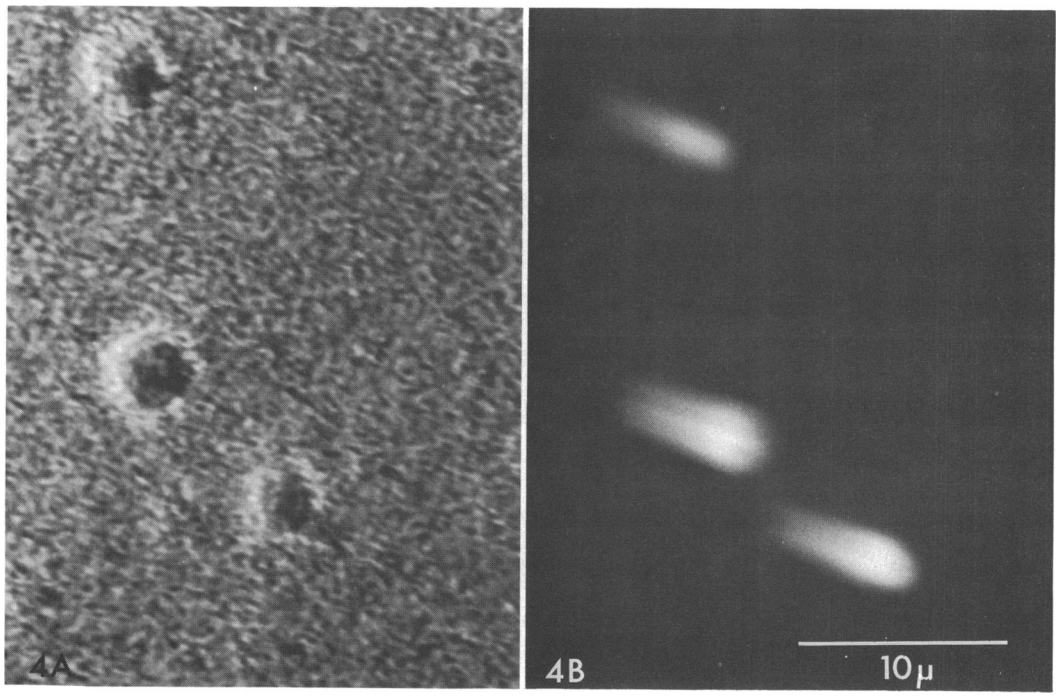

FIG. 4. *Dictyostelium sp. (4a) Phase contrast. Note the dead bacteria on which the slime mold is growing. (4b) Fluorescence. The comet-like appearance is due to the movement of the cells during the exposure.*

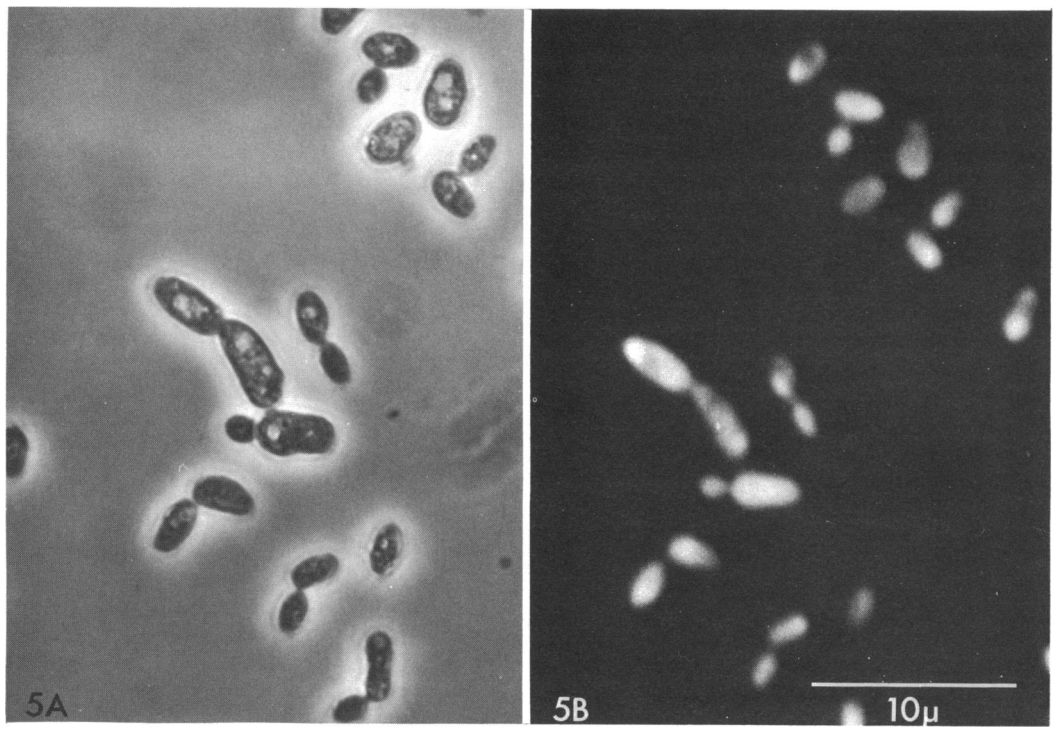

FIG. 5. *Lipomyces lipofer.* (*5a*) *Phase contrast. The refractile inclusions correspond to the lipid inclusions as judged by oil red O and Sudan black B staining.* (*5b*) *Fluorescence.*

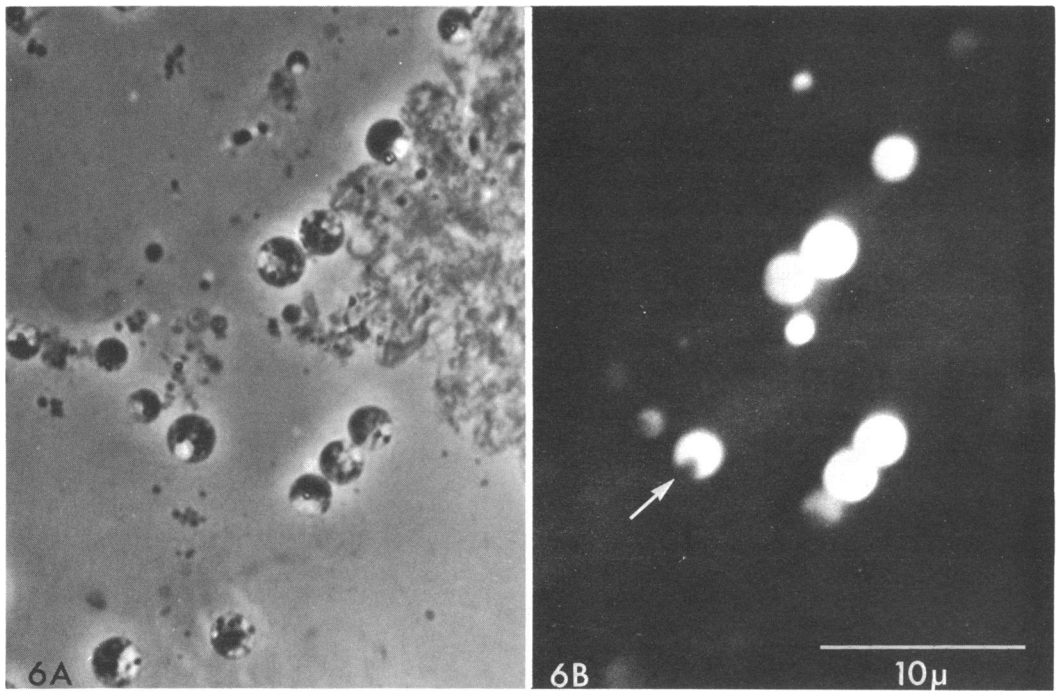

FIG. 6. *Lipomyces lipofer protoplasts.* (*6a*) *Phase contrast; the lipid droplets are evident.* (*6b*) *Fluorescence; the fluorescein does not localize within the lipid droplets* (*arrow*).

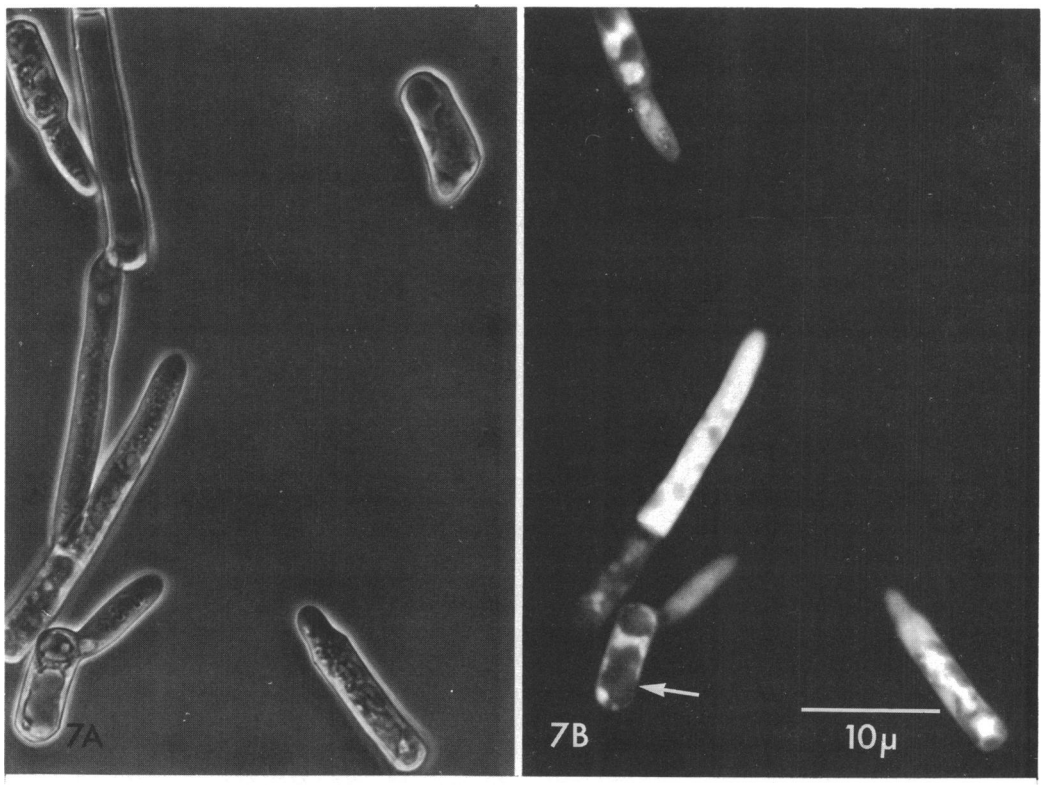

FIG. 7. *Endomyces magnusii. (7a) Phase contrast; (7b) fluorescence. Note the concentration of fluorescein in the actively growing cell; the cell with retracted cytoplasm (arrow) shows little fluorescence.*

the organisms. Because the cells were living and many were actively motile, photographs requiring a 3-min exposure time were sometimes impossible to obtain. Quenching of fluorescence was a second problem. In many cases, upon exposure to ultraviolet radiation for periods over 1 or 2 min, the glow began to decrease and was soon extinguished. The inherent difficulties in using fluorescent microscopy to observe cells have been reviewed by Darken (7) and deRepentigny (24).

The findings of Rotman and Papermaster (29) support the concept that the FDA which is relatively nonpolar can diffuse through the membrane into the cell, where the fluorescein evolved by esterolytic enzymes cannot leave the cell, owing to its polarity (*see* Fig. 12). In this study, we are concerned with the passage of the substrate through a slime layer, cell wall, and cell envelope in order to reach the enzyme. The fact that this does occur is evident from the fluorescent photomicrographs presented here. When the rate of enzyme activity in the intact cell is compared with that of the protoplast or spheroplast, it is found that the wall does present a barrier to the efflux of the liberated fluorescein. This was evident when attempts were made to photograph protoplasts of organisms other than *L. lipofer*. In data to be presented elsewhere (Medzon and Brady, *in preparation*), it is shown that the rate of substrate uptake and hydrolysis in living bacteria is a function of the environment during the assay. The complexity of the microbial cell surface (21) and the fact that esterase activity can vary over the life cycle of an organism (33) or during intracellular digestion (20) might also be an explanation for the absence of acetylesterase activity in some organisms studied.

The unique finding of this study was the concentration of the fluorescein end product in membranes or membrane-bounded organelles. Esterase activity has been reported in the food vacuoles of protozoa (11, 19). The presence of acid phosphatase and other enzymes in food vacuoles has prompted the inclusion of these bodies within the definition of a lysosome (20). In this study, the presence of esterase activity in the vacuoles was especially evident in *Acanthamoeba* sp., of which the fine structure has been clearly described (4). The presence of acetylesterase within the nuclear region and perhaps in the pyrenoids of green algae fits in well with the findings of Hunter (13) and of Manton (16).

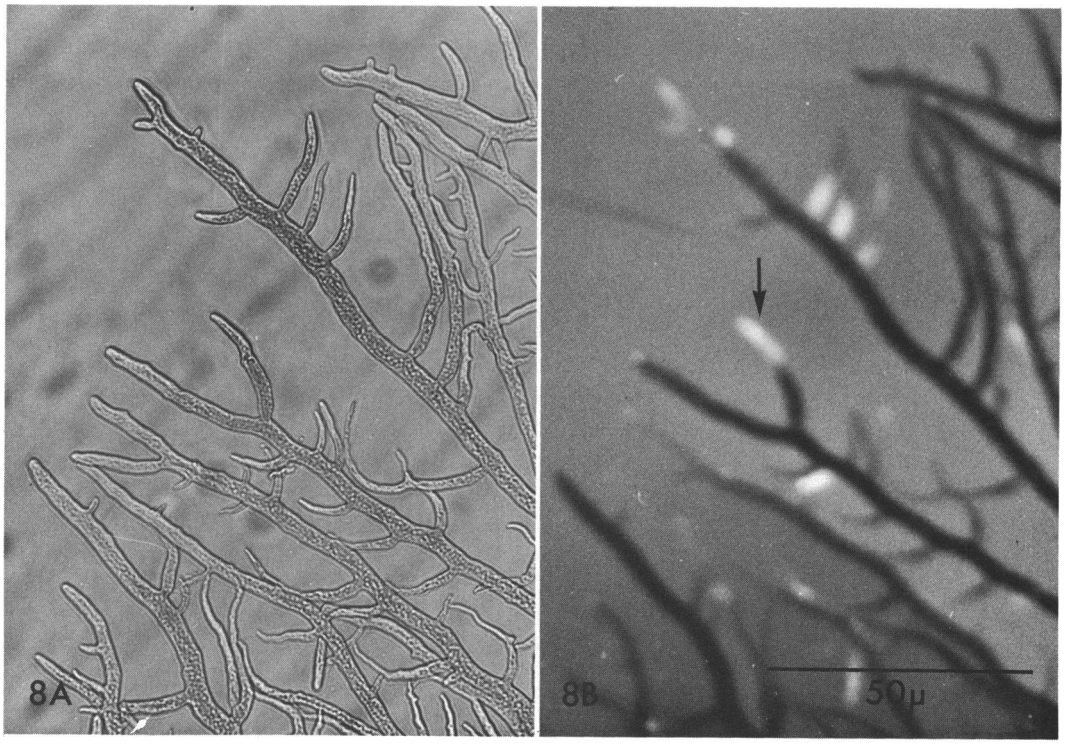

FIG. 8. *Absidia spinosa.* (*8a*) *Phase contrast;* (*8b*) *fluorescence. Note the concentration of fluorescein in the hyphal tips. The bright background is due to fluorescein released into the medium.*

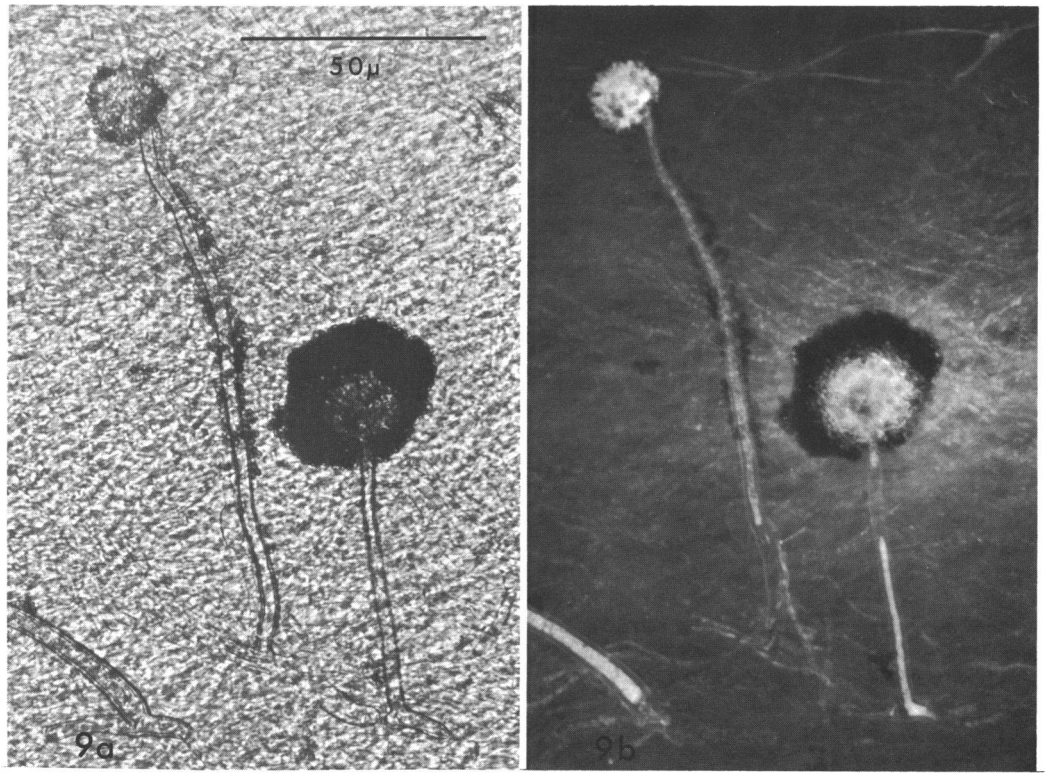

FIG. 9. *Aspergillus niger fruiting body.* (*9a*) *Phase contrast;* (*9b*) *fluorescence. The fluorescein is concentrated in the less mature spores. The foot cell and the internal cytoplasm are also stained.*

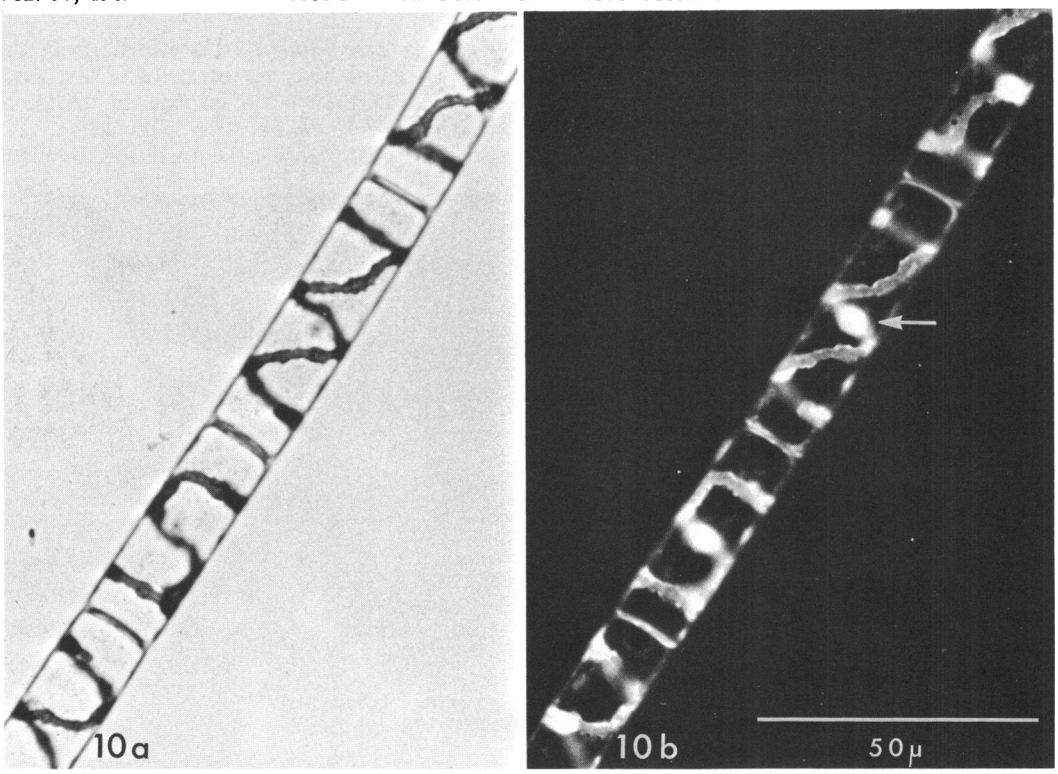

FIG. 10. *Spirogyra sp.* (*10a*) *Ultraviolet absorbance. Note the lack of absorbance by the nucleus.* (*10b*) *Fluoresence. The helical chloroplast autofluoresces red. The fluorescein is concentrated in the nuclear region* (*arrow*) *and along the membrane of the chloroplast and the cell.*

The observation that fluorescein concentrates in the growing hyphal tips of fungi and then spreads throughout the mycelium is reminiscent of the findings of Schütte (32), except that the concentration of fluorescein used in his study was higher than that used here. In the case of yeast, the observation that the lipid droplet of *L. lipofer* excludes the fluorescein follows the hypothesis of Rotman and Papermaster (Fig. 12). Our observations of the concentration of enzyme within vacuoles fits the data presented by Matile and Wiemken (17), who refer to the yeast vacuole as a lysosome. Pitt (23) has shown esterase activity in particles within the hyphae of *Botrytis cinea*, which he refers to as analogues of lysosomes. We have seen similar particles in several fungi.

In the bacteria studied, a most interesting finding was the apparent concentration of fluorescein in a mesosome-like region (8) in a young growing culture of *B. megaterium*. Recently, esterase activity has been demonstrated in these regions by means of electron microscopy (3, 36). The location of esterase activity in sporulating cells was also demonstrated. This is also consistent with our findings.

The location of acetylesterase activity within the cell is complicated by the evidence presented in the review by Salton (30) in which several enzyme activities appear to be on the outside of the cell membrane.

Esterase activity has also been shown in extracellular fluids of streptococci (35), whereas it has been shown that an aliesterase is associated with the cytoplasm but not with the membrane fraction of bacterial protoplasts (31). This latter finding is consistent with data (Medzon and Brady, *in preparation*) which show that supernatant fluids from stored protoplasts did not demonstrate large amounts of acetylesterase activity relative to the intact protoplast.

From this survey, it is apparent that the use of fluorescein diacetate as an acetylesterase substrate provides a unique opportunity to study the permeability of nonpolar compounds into cells which have a variety of surface structures, as well as to define by light microscopy the general regions in which enzyme activity appears to be located. The method is rapid and direct and does not require added electron acceptors to localize the end product. The fact that microorganisms are

not killed by the substrate enables one to measure the enzyme activity directly on living cells in a fluorometer. The kinetics of the enzyme activity and the penetration of inhibitors into cells is the subject of another communication (Medzon and Brady, *in preparation*). It is evident from the data presented here, however, that one cannot easily classify the types of enzymes by the use of classical esterase inhibitors on living cells.

Of great importance is the use of this technique

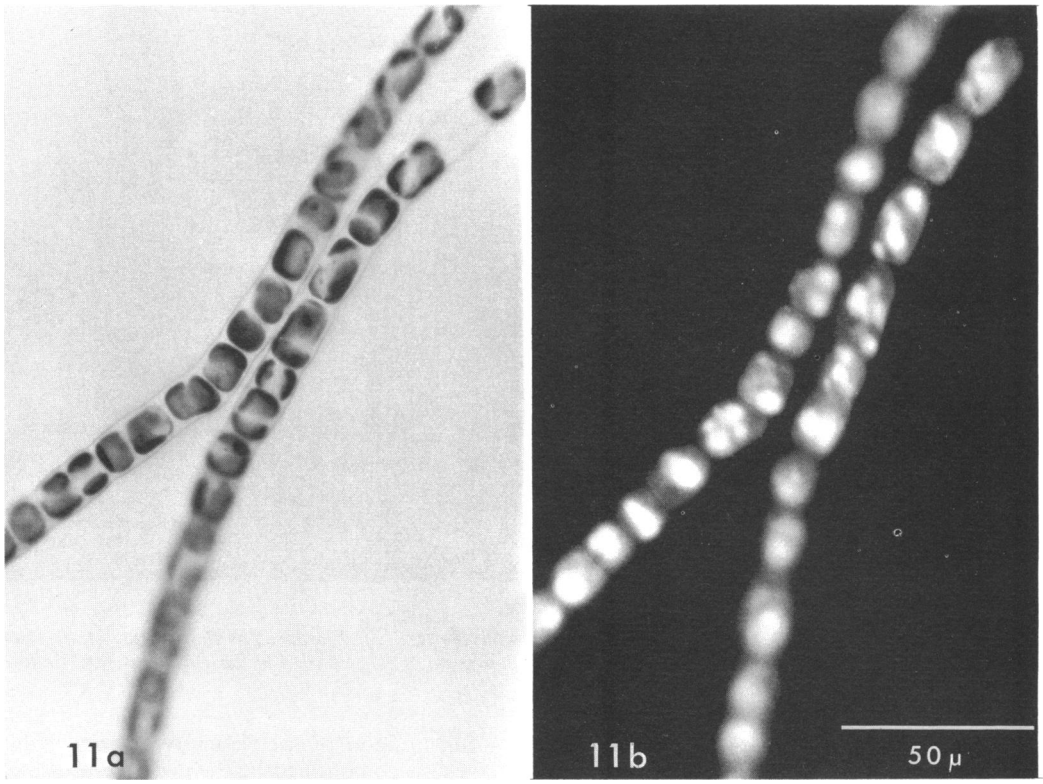

FIG. 11. *Tribonema aequale. (11a) Ultraviolet absorbance. Note the diverse structure of the chloroplast. (11b) Fluorescence. Note that the chloroplasts appear to surround the bright fluorescein-stained nuclear region.*

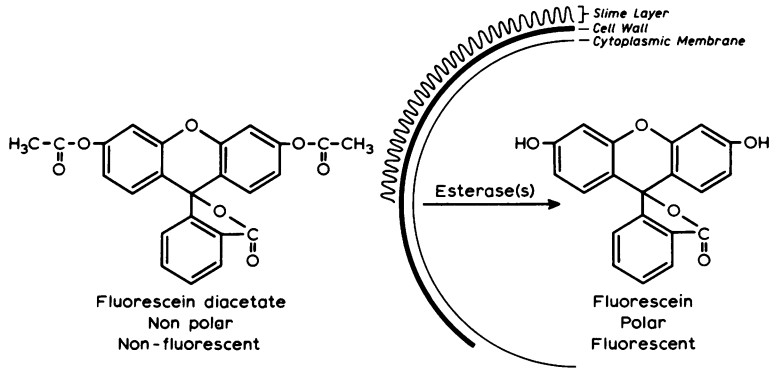

FIG. 12. *Proposed mechanism for the fluorochromatic reaction of living cells with the acetylesterase substrate, fluorescein diacetate, modified from Rotman and Papermaster (31). Surface layers of microbial cells, as defined by Salton (30), are superimposed. Intracellular organelles which concentrate the end product, fluorescein, are not shown, but are assumed to follow the same mechanism. The fate of acetate is unknown.*

TABLE 3. *Inhibition of acetylesterase activity in living microorganisms by 4.9 × 10^{-5} paraoxon*

Organism	Cells per milliliter	Inhibition
		%
Bacillus megaterium.....	15×10^6	100
Spirillum serpens.....	32×10^6	100
Diplococcus pneumoniae...	12×10^6	38
Saccaromyces cerevisiae...	3.5×10^6	32
Lipomyces lipofer.....	4.4×10^6	40

in demonstrating to students the dynamics of enzyme activity in living organisms. Exercises based on these studies have been utilized in this department for 2 years.

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