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THE AUTOFLUORESCENT "LIPOFUSCIN GRANULES" IN THE INTESTINAL CELLS OF *CAENORHABDITIS ELEGANS* ARE SECONDARY LYSOSOMES

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SUMMARY

The nematode *Caenorhabditis elegans* contains autofluorescent lipofuscin granules, located exclusively in the 32–34 intestinal cells. Using epifluorescence microscopy on live adult animals, we have shown that fluorescent-labeled exogenous probes are taken up by endocytosis and accumulate within the granules. Macromolecular solutes such as proteins and dextran appear to be taken up by fluid-phase pinocytosis. There is no phagocytosis of latex particles with diameter $\geqslant 0.25$ μm. The granules concentrate the lysosomotropic weak base acridine orange, indicating that they have an acidic internal milieu. These observations imply that the lipofuscin granules in the intestinal cells are secondary lysosomes which remain active recipients of endocytosed materials.

Key words: Lipofuscin; Autofluorescence; Endocytosis; Lysosomes; Nematodes; *Caenorhabditis elegans*

INTRODUCTION

The free-living nematode *Caenorhabditis elegans* is finding increasing use as a model system for the study of aging phenomena (for review see refs. 1,2). In large part, this choice has been motivated by the favorable genetic properties of the organism [3], which facilitate the isolation and characterization of mutant strains [4] and recombinant inbred lines [5] with altered lifespans and/or altered rates of senescent changes.

A limited characterization of senescent changes in *C. elegans* has suggested some parallels with senescent changes in mammals. Both longitudinal and populational studies have revealed a regular increase in probability of mortality [6,7], regular declines in behavioral or neuromuscular functions [6–8], and a decline in fertility [6]. Restriction of food intake increases the lifespan of *C. elegans* [6], as it does for mammals.

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With increasing age, nematodes accumulate fluorescent pigments [6] whose solubility properties and fluorescence spectra [9] closely resemble those of the "lipofuscin" pigments [10] which accumulate in the tissues of aging vertebrates. Osmophilic materials which resemble "lipofuscin granules" [11] accumulate in the intestinal epithelium of nematodes [12,13]. Histochemical staining suggested that these granules contain acid phosphatase [12], which is a lysosomal enzyme in nematodes [14].

We report here that the autofluorescent "lipofuscin granules" in the intestinal cells of *C. elegans* share two further properties of secondary lysosomes: they accumulate exogenous materials taken into the cells by endocytosis and they concentrate a lysosomotropic weak base. Our observations indicate that the "lipofuscin granule" compartment is not physiologically inert, but may be the recipient of a variety of dietary (or cellular) constituents.

MATERIALS AND METHODS

Preparation of fluorescent macromolecular conjugates

To prepare rhodamine B isothiocyanate (RITC) conjugates of bovine serum albumin (BSA) or rabbit immunoglobulin G (IgG), 200 mg of either protein was dissolved in 2 ml of reaction buffer (50 mM sodium carbonate/bicarbonate, pH 8.6). The solution was stirred for 40 min at room temperature with 36 mg of 10% RITC on Celite. The Celite was removed by two successive centrifugations for 20 min at 1000 *g*. To separate the conjugate from unbound fluorophore, the solution was passed through a column (5 ml bed volume) of Sephadex G-25, packed and eluted with 10 mM ammonium acetate (pH 7). The first of two well-resolved pink bands emerging from the column contained RITC-protein conjugate. This was lyophilized twice (with an intervening water wash) to remove ammonium acetate, and the dry product stored desiccated at −20°C. For use, the RITC-protein was redissolved at a final concentration of 10 mg/ml in BU buffer [72 mM potassium phosphate (pH 7); 70 mM NaCl]. This stock solution was stored at 5°C and used within 2 weeks.

The same method was used to prepare RITC-dextran, except that the reaction proceeded for 24 h at pH 7.5. We found it necessary to heat (95°C, 5 min) solutions of both unconjugated and conjugated dextran to achieve complete solubilization.

To remove sodium azide from commercial preparations of tetramethylrhodamine isothiocyanate-concanavalin A (RITC-ConA), we passed a solution (10 mg/ml) through a column of Bio-Gel P2, packed and eluted with 10 mM ammonium acetate (pH 7). The conjugate was lyophilized and stored as described above. Stock solutions of RITC-ConA were prepared in HB buffer (75 mM HEPES (pH 7); 100 mM NaCl; 1 mM $CaCl_2$; 1 mM $MnCl_2$).

The fluorescent monodisperse carboxylated microspheres (mean diam. 0.25 μm) were received as a suspension of 2.5% solid in water. To clean them, 1 ml of the suspension was added to 1 ml of 100% ethanol, centrifuged for 5 min at 15 000 *g* and the supernatant removed. The pelleted microspheres were re-suspended in 2 ml of 50%

aqueous ethanol and re-centrifuged. The supernatant was removed and the pelleted microspheres were allowed to dry overnight. They were then re-suspended in 1 ml BU buffer. The re-suspended stock was stored at 5°C and used within 2 weeks. The microspheres were never frozen.

Growth of nematodes

A wild-type strain of *Caenorhabditis elegans* (strain N2, also known as the Bristol variety [3]) and *Escherichia coli* strain OP-50 (a uracil auxotroph) were originally obtained from the collection of R.L. Russell. Cultures of *C. elegans* were grown monoxenically at either 25°C or 16°C on NG agar plates [3] seeded with a lawn of *E. coli*. Age-synchronous cultures were established by placing 10–20 adult hermaphrodites onto fresh plates, allowing them to lay eggs for 2 h (at 25°C) or 4 h (at 16°C), then removing the adult worms from the plate. The ages of the experimental animals are given from the middle of the egg-laying period. Each plate contained a few hundred animals, which neither crowded the plate nor exhausted the food supply (*E. coli*) in the first generation.

Feeding of the conjugates

Age-synchronous *C. elegans* were fed various fluorescent-labeled probes or unlabeled control probes at a fixed developmental stage defined by the start of their egg laying period. This stage was attained at 90 h for worms raised at 16°C or at 65 h for worms raised at 25°C [cf. ref. 15].

A 50-μl drop of the feeding solution was placed in the inverted lid of a Petri dish and 25–50 worms were transferred to the drop. No attempt was made to prevent the transfer of *E. coli* with the worms. The bottom of the Petri dish with NG agar and an *E. coli* lawn was then placed over the inverted lid as a humidifier. After the specified feeding period, the worms were washed in 1 ml of the feeding buffer (without the fluorescent probe or unlabeled control macromolecule) and collected by low speed centrifugation, (500 *g* for 5 min). The worms were transferred to the periphery of an NG agar plate with an *E. coli* lawn and allowed to feed on the plate for 4 h, during which time most unincorporated probe was excreted.

In experiments using RITC-ConA, the worms were fed and flushed in the same manner except for the following changes: the feeding and washing buffer was HB buffer. After feeding, the worms were not placed on an *E. coli* lawn, but were flushed by incubation in HB buffer with live *E. coli* (approx. 10^8 cells/ml).

Fluorescence microscopy

After feeding and flushing, the live worms were placed on standard microscope slides for observation. A 10–14-μl drop of BU buffer or HB buffer was placed on a glass slide and 10–20 worms were quickly placed in the drop. An 18 mm × 18 mm glass coverslip was lowered gently over the drop. The slide was then checked with a dissecting microscope to see if the worms were properly mounted. If the proper amount of buffer had been used the worms were trapped firmly between the cover slip and slide. If too little

buffer were used, the worms would burst. If too much were used, the worms would move too fast for photomicrography. Adjustments in volume could be made by adding a few microliters of buffer with a micropipet or withdrawing a few microliters with a tissue. After proper mounting, the edges of the coverslip were sealed with a small drop of low-fluorescence immersion oil.

We used a Zeiss Universal microscope with a Zeiss C-35 camera and photographic attachment, Zeiss vertical illuminator III-RS epi-illumination fluorescence attachment, and Zeiss Neofluor objective lenses. The four combinations of filters in the epi-illumination barrier and exciter sliders were as follows:

1. For observation of autofluorescence, we used Zeiss filter set 02, consisting of exciter filter G365 (excitation 325–375 nm; peak 365 nm), dichromatic beam splitter FT395 and barrier filter LP420 (emission, 50% transmission at 420 nm). This combination is referred to as 365 → 420 epi-illumination.
2. For observation of acridine orange, we used Zeiss filter set 03, consisting of exciter filter BP405/14 (excitation 390–420 nm; peak 405 nm), dichromatic beam splitter FT425 and barrier filter LP435 (emission, 50% transmission at 435 nm). This combination is referred to as 405 → 435 epi-illumination.
3. For observation of FITC conjugates, we used Zeiss filter set 11, consisting of exciter filter BP450–490 (excitation 450–490 nm; peak 480 nm), dichromatic beam splitter FT510 and barrier filter LP520 (emission, 50% transmission at 520 nm). This combination is referred to as 480 → 520 epi-illumination.
4. For observation of RITC conjugates, we used Zeiss filter set 14, consisting of exciter filter BP515–560 (excitation 515–560 nm; peak 546 nm), dichromatic beam splitter FT580 and barrier filter LP590 (emission, 50% transmission at 590 nm). This combination is referred to as 546 → 590 epi-illumination.

Photography was done using Kodak high-speed (ASA 400) Ektachrome film, type EL. Commercial vendors developed the film. The color slide images were then transferred to black and white print film, Polaroid Type 665, using a Polaroid Polaprinter slide copier.

Photographic exposures for the initial color slides were 1–2 min for epifluorescence illumination and 12 sec for transmitted white-light illumination.

Chemicals and biochemicals

The following reagents were purchased from commercial sources: acridine orange from Biomedical Specialties Co., Los Angeles, CA; bovine serum albumin (fatty acid and globulin free, RIA grade) and yeast α-mannan from Sigma Chemical Co., St. Louis, MO; Dextran-10 (mol. wt 10 000) from Pharmacia, Uppsala, Sweden; fluorescent (fluorescein conjugated) monodisperse carboxylated microspheres (0.25 μm diameter) from Polysciences Inc., Warrington, PA; Rhodamine-B isothiocyanate (10% on Celite) from United States Biochemicals Co., Cleveland, OH; and RITC-ConA from Vector Labs. Inc., Burlingame, CA.

RESULTS

Autofluorescent "lipofuscin" granules in C. elegans

As observed under epifluorescence illumination (365 → 420 nm), *C. elegans* contains a population of blue autofluorescent granules (Fig. 1) which are limited to the 32–34 intestinal cells. The number of such granules, which we estimate as several hundred per cell, appeared approximately constant from cell to cell. Within each cell, the granules were distributed throughout the cytoplasm but excluded from the nuclei, which are seen in Fig. 1 as dark areas. The positions of the granules within the cells are not fixed, but vary from animal to animal.

The sizes of the granules varied from approximately 0.5 to 3 μm, consistent with the size distribution of the osmophilic "lipofuscin" granules [12] and the size distribution expected for a population of secondary lysosomes [16].

Loading of the granules with macromolecular probes

To determine whether these granules, like secondary lysosomes, were the repositories for materials taken up by endocytosis, we fed living animals a variety of macromolecules conjugated to a fluorophore. As shown in Fig. 2, *C. elegans* readily took up such probes (in this case RITC-BSA) and sequestered the material in granules in the intestinal cells. In the original color photographs these granules were brilliant red, indicating rhodamine fluorescence. In all animals, the fluorescent probe was observed only in these intestinal granules, with the minor exception of some diffuse fluorescence in the buccal and pharyngeal linings and the spermathecae.

By comparing, in each individual cell, the patterns of granules loaded with RITC-BSA to the pattern of blue autofluorescent granules (Fig. 2), we determined that all blue autofluorescent granules had been loaded with RITC-BSA. There was usually a minority population of granules which contained red fluorescence but not blue autofluorescence. We estimate that this group constitutes less than 5% of the total number of granules. Thus, the patterns of blue and red granules in each cell are nearly, but not completely, identical. Similar observations have been made with RITC-dextran (Fig. 3) or RITC-IgG as probes.

The apparent number and size distribution of blue autofluorescent granules appeared the same in animals taken directly from feeding plates without incubation in buffer, in animals fed rhodamine-conjugated proteins, and in animals fed unconjugated BSA or IgG under the same feeding regimen in buffer. Furthermore, we obtained the same results when animals were fed on agar plates with *E. coli* lawns overlaid with RITC-BSA, or when *E. coli* (1×10^8 cells/ml) was added to the buffered solution of RITC-BSA for feeding experiments in liquid. These experiments demonstrate that neither the feeding regimen nor the protein probes themselves cause redistribution of the blue autofluorescence or apparent swelling of the granules.

By contrast, in the case of animals fed RITC-dextran (Fig. 3) or unconjugated dextran (not shown), there was a noticeable shift of the granules to larger average size. In parti-

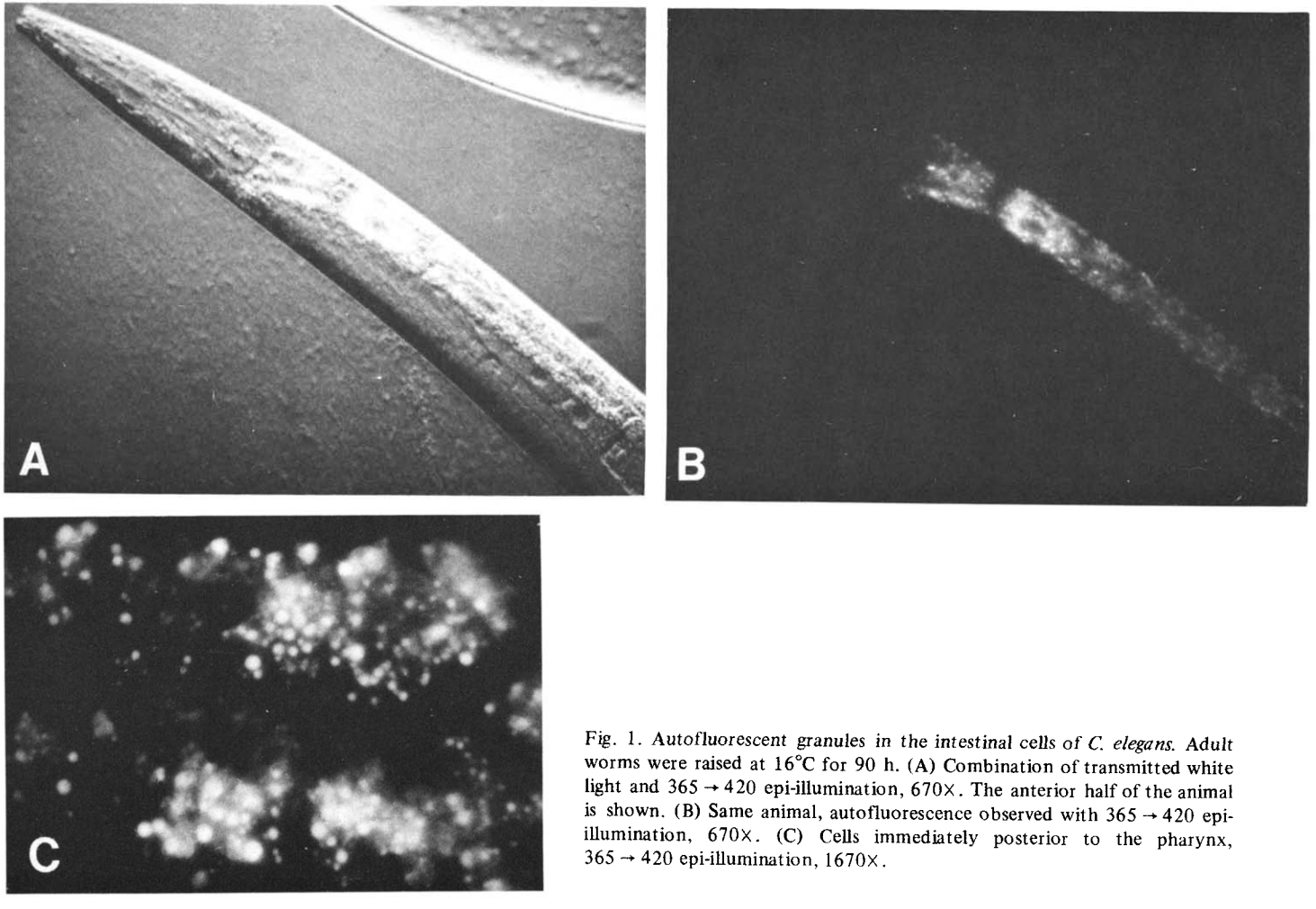

Fig. 1. Autofluorescent granules in the intestinal cells of *C. elegans*. Adult worms were raised at 16°C for 90 h. (A) Combination of transmitted white light and 365 → 420 epi-illumination, 670×. The anterior half of the animal is shown. (B) Same animal, autofluorescence observed with 365 → 420 epi-illumination, 670×. (C) Cells immediately posterior to the pharynx, 365 → 420 epi-illumination, 1670×.

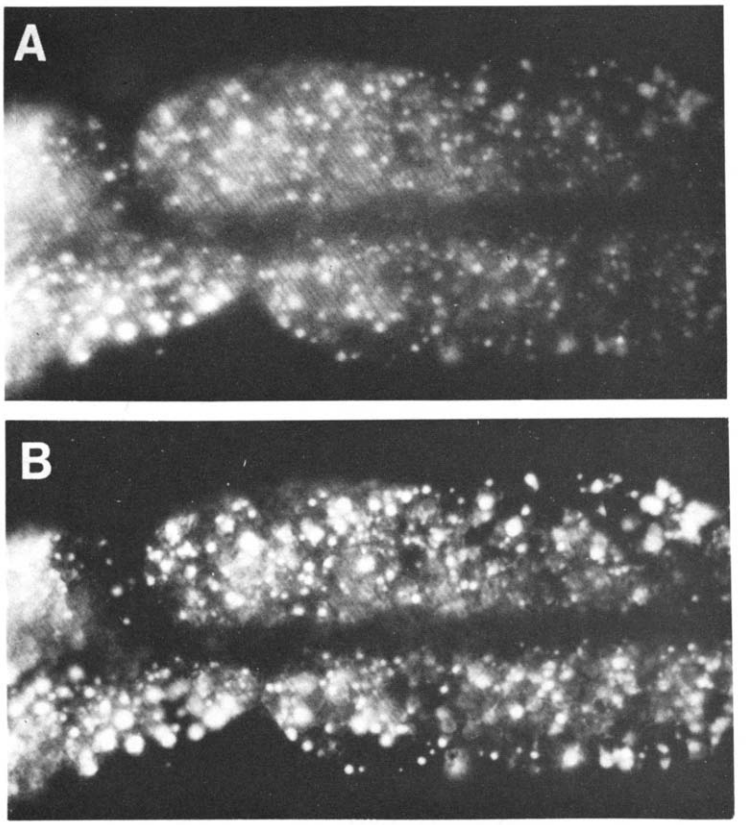

Fig. 2. Coincidence of autofluorescence and ingested RITC-BSA in intestinal granules. Animals raised at 16°C to 90 h. of age were fed RITC-BSA for 8 h, then "chased" for 4 h on lawns of *E. coli* OP50. (A) Cells immediately posterior to the pharynx, autofluorescence (blue) viewed with 365 → 420 epi-illumination, 1670×. (B) Same view, but RITC-BSA fluorescence (red) viewed with 546 → 590 epi-illumination, 1670×.

cular, the population of smaller granules which appeared in control animals or animals fed on protein solutions was greatly depleted after feeding of dextrans (cf. Fig. 2 to Fig. 3), although the total number of granules per cell was about the same. This was equally apparent in both the blue autofluorescence and the red fluorescence due to RITC-dextran (see Fig. 3), further implying that the same population of granules was swelling in response to the ingestion of dextran.

After the granules had been loaded with probes such as RITC-BSA, a prolonged "chase" period on an *E. coli* lawn in the absence of probe led to no significant redistribution of the probe into another cellular compartment. Thus, the granules represent the ultimate cellular destination of such materials taken up from the intestinal lumen, rather than an intermediate compartment through which the materials pass.

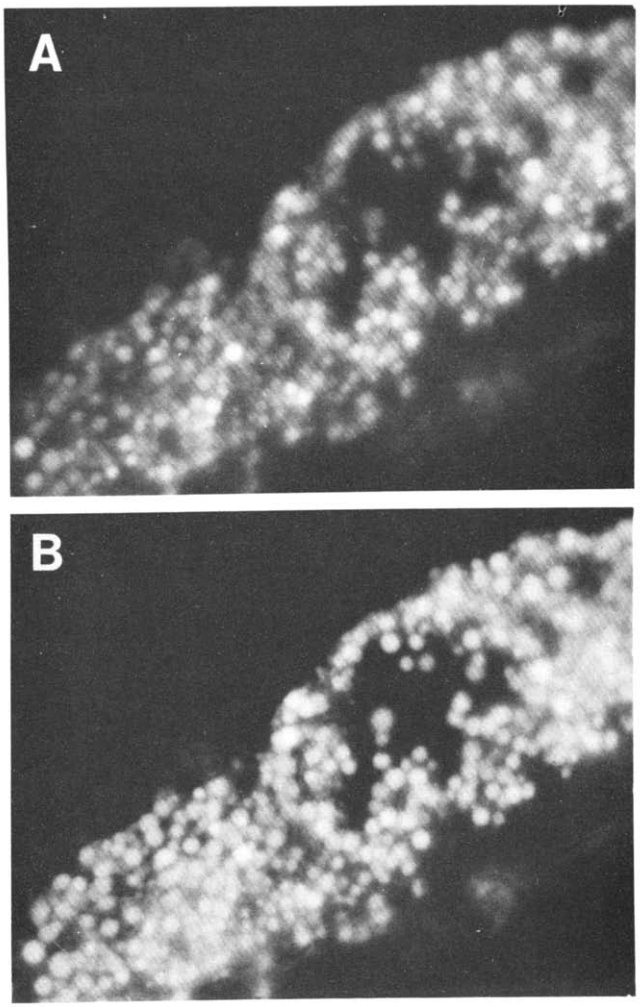

Fig. 3. Coincidence of autofluorescence and ingested RITC-dextran in intestinal granules. Growth and feeding protocol as for Fig. 2, except that RITC-dextran was used. (A) Cells immediately posterior to the pharynx, autofluorescence (blue) viewed with 365 → 420 epi-illumination, 1670×. (B) Same view, but RITC-dextran fluorescence (red) viewed with 546 → 590 epi-illumination, 1670×.

Delocalization of autofluorescence upon cell death

The autofluorescent pigments did not seem to be associated with only the membrane of the granules. Upon prolonged observation in the microscope, we noted that dying animals released the blue autofluorescent material from the granules, such that individual cells became uniformly blue. Incorporated RITC-BSA, by contrast, remained localized in the granules. This does not necessarily imply that the membranes surrounding the granules remained intact, since RITC-BSA might have been precipitated *in situ* by the acidic conditions found within the granules (see below).

Uptake of probes by fluid-phase pinocytosis

Three lines of evidence indicate that the fluorescent probes are taken up by fluid-phase pinocytosis rather than by receptor-mediated endocytosis or phagocytosis. First, we found that adding a 10-fold excess (1% w/v) of unconjugated BSA to the feeding solution (0.1% RITC-BSA in buffer) had no apparent effect on the rate of uptake of the RITC-BSA. If uptake had been mediated by saturable receptors on the luminal surfaces of the intestinal cells, the excess unlabeled BSA should have inhibited uptake of RITC-BSA by competing for binding sites.

Second, we have noted that the amount of fluorescent material taken into the cells and accumulated in the granules increases with increasing concentration of probe in the feeding solution. Although the thickness of the intestinal cells and the spurious adsorption of probe to the buccal and pharyngeal linings have so far prevented quantitative measurement of uptake rates, these semi-quantitative observations are also consistent with fluid-phase uptake.

Third, we fed living *C. elegans* fluorescein-labeled latex microspheres (0.25 μm mean diameter) under the same feeding conditions. Although the microspheres were readily ingested into the intestinal lumen (Fig. 4), there was no incorporation of any microspheres into the intestinal cells. We have also seen no indication, in examining electron micrographs of thin sections of *C. elegans*, that large particles of the normal bacterial food enter the intestinal cells (O.J. Bashor and L.A. Jacobson, unpublished observations). We doubt that these cells are capable of phagocytosis.

The weight of evidence therefore indicates that macromolecules, taken up from the intestinal lumen by fluid-phase pinocytosis, find their ultimate intracellular destination in the blue autofluorescent "lipofuscin granules" of the intestinal cells. It seems to us unlikely that degradation of the macromolecules is prerequisite to their uptake, since the presumably inert RITC-dextran is taken up about as well as RITC-proteins and has the same subcellular compartment as its destination. The only exceptional case we have yet found is RITC-ConA, a lectin which binds tenaciously to the luminal surface and is never taken up into the cells.

Accumulation of a lysosomotropic agent

To determine if the blue autofluorescent granules resembled lysosomes in having an acidic internal environment, we fed the living animals solutions of acridine orange. This lysosomotropic weak base passes readily through membranes in its unprotonated form, but cannot pass back out through the lysosomal membrane after protonation in the acidic interior of the lysosomes [17,18]. This leads to a net concentration of the dye in the lysosomes, with a resulting red shift in fluorescence emission. Thus, depending upon the absolute concentration achieved, acridine-loaded lysosomes fluoresce yellow to orange-red.

As shown in Fig. 5a, *C. elegans* took up acridine orange after feeding in relatively concentrated (0.1 mg/ml) solution. Distinct yellow to orange staining was localized only in granules in the intestinal cells. Weaker green to yellow-green staining, probably due

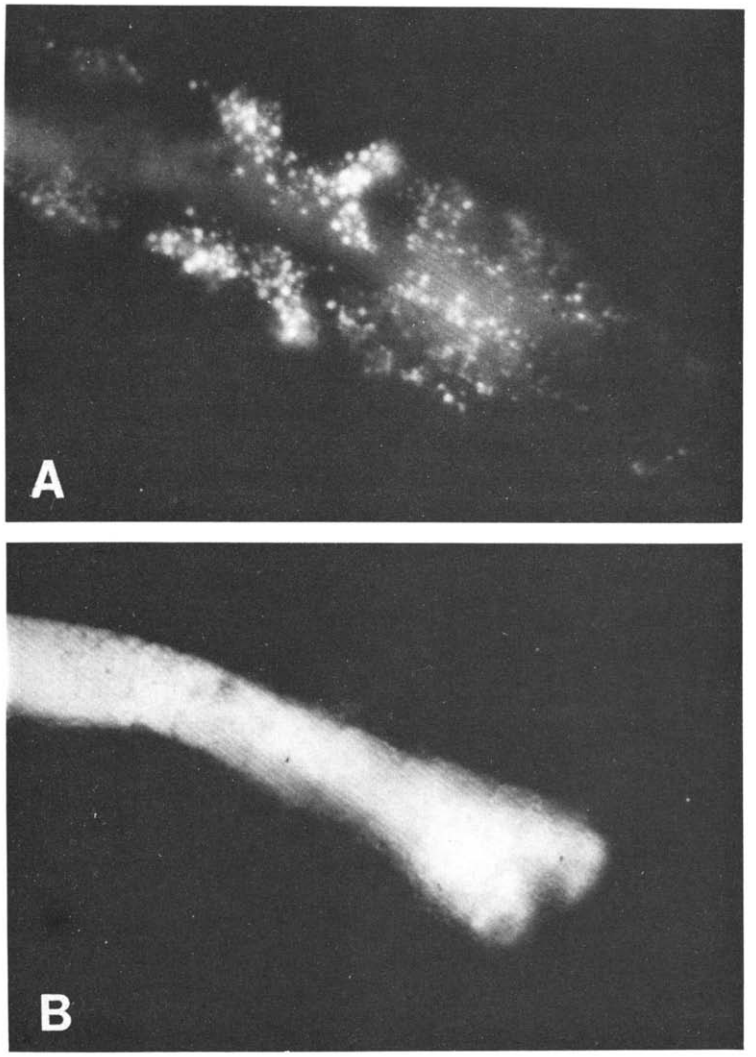

Fig. 4. Latex microspheres do not enter the intestinal cells. Adult animals (90 h old at 16°C) were fed a 1.25% suspension of FITC-latex microspheres for 8 h, then flushed for 4 h on lawns of *E. coli* OP50. (A) Autofluorescence in the cells of the anterior intestine, autofluorescence (blue) viewed with 365 → 420 epi-illumination, 1070×. (B) Same view, but FITC-microspheres (0.25 μm diameter), limited to the intestinal lumen, viewed with 480 → 520 epi-illumination, 1070×.

to association with nucleic acids, was visible in the gonad, the pharynx and the spermathecae. At this concentration, the broad-band fluorescence of acridine orange completely masked the blue autofluorescence of the intestinal granules. The fact that we did not observe a separate population of blue granules suggested that all blue autofluorescent granules had accumulated the acridine dye.

To confirm this directly, we fed another group of animals a more dilute (0.01 mg/ml)

acridine orange solution. The resulting lower concentration of dye in the lysosomes permits direct comparison of the granules containing acridine orange (480 → 520 epifluorescence illumination, Fig. 5c) to those containing blue autofluorescence (365 → 420 epifluorescence illumination, Fig. 5b). Identical patterns were observed, indicating that all blue autofluorescent granules had accumulated acridine orange.

DISCUSSION

Nematode lipofuscin resembles mammalian lipofuscin

Although it appears likely that the term "lipofuscin" denotes a class of heterogeneous

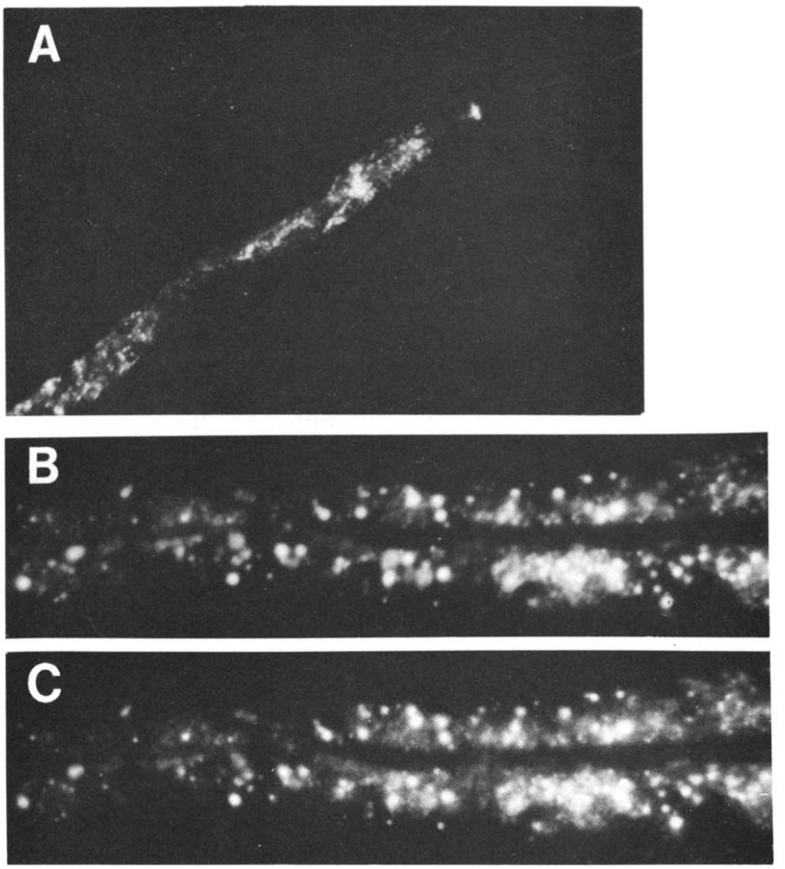

Fig. 5. Coincidence of autofluorescent granules with granules loaded by acridine orange. Adult animals (90 h old at 16°C) were fed acridine orange in solution for 8 h, then flushed for 4 h on lawns of *E. coli* OP50. (A) Worm fed acridine orange at 0.1 mg/ml, intense orange fluorescence limited to the intestinal granules, viewed in the anterior intestine with 405 → 435 epi-illumination, 670×. (B) Worm fed acridine orange at 0.01 mg/ml, autofluorescence (blue) viewed with 365 → 420 epi-illumination, 1070×. (C) Same view, fluorescence of dilute acridine orange (yellow) viewed with 480 → 520 epi-illumination, 1070×.

substances rather than a single chemical substance, the blue autofluorescent material found in the intestinal cells of *C. elegans* resembles the "lipofuscin" pigment of mammalian cells by several criteria:

1. Both nematode and mammalian lipofuscins are soluble in lipid solvents such as chloroform-methanol [9].
2. After extraction into lipid solvents, the fluorescence spectra of nematode lipofuscins [9] closely resemble those of mammalian lipofuscins [10].
3. In nematodes, as in mammals, the cellular content of lipofuscin increases with age [6,9]. Nematode lipofuscin shows the same age-dependence when measured fluorimetrically after extraction into chloroform-methanol or chloroform-isoamyl alcohol or, alternatively, by direct *in situ* microfluorimetric measurements on individual cells in the posterior region of the intestine (C.M. Link, R.L. Russell and L.A. Jacobson, unpublished observations).
4. The characteristic blue autofluorescence in the intestinal cells is localized in discrete granules, the size and localization of which closely resemble those of the osmophilic "lipofuscin granules" observed in related nematodes by electron microscopy [12,13]. Mammalian lipofuscins are localized in morphologically similar granules [19], although their distribution clearly includes a wider variety of cell types.

Thus, although complete chemical characterization is available for neither nematode nor mammalian lipofuscins, they appear to be similar substances (or classes of substances) which accumulate with age in similar subcellular compartments.

The lipofuscin granules are secondary lysosomes

Previous enzyme histochemical studies [12] of nematode lipofuscin granules had provided suggestive evidence that they are lysosomes. In particular, the granules were shown to contain acid phosphatase activity, which behaves as a lysosomal marker in *C. elegans* during subcellular fractionation [14]. So far as we know, no other lysosomal marker enzymes have been demonstrated in these osmophilic granules.

Analogous attempts to identify mammalian lipofuscin granules as lysosomes by enzymatic criteria have led to contradictory interpretations. For example, lipofuscin granules with morphological similarity to lysosomes have been shown by histochemical methods to contain not only acid phosphatase [19,20], but also β-glucuronidase and β-hexosaminidase activities [21]. On the other hand, lipofuscin granules purified from human cardiac muscle cells were not enriched for acid phosphatase or cathepsin [11,22]. Neither acid phosphatase nor esterase activities co-purified with the main mass of lipofuscin granules, but were found in a slower-sedimenting peak with a lower content of lipofuscin pigment [11].

Both enzyme histochemical methods and subcellular fractionation techniques have potential limitations. The chemical fixations which precede histochemical detection of

enzyme activity may produce artifactual localizations or preferential survival of activity in particular compartments [22]. A further consideration is that acidic lipofuscins may simply adsorb enzymes [22]. Our observation that the blue autofluorescence is rapidly delocalized upon cell death also suggests caution in the interpretation of results obtained by subcellular fractionation, which is in any event technically difficult in nematodes because of the harsh conditions necessary to disrupt the tough exterior cuticle [14].

We have therefore selected two properties of lysosomes which can be evaluated qualitatively by non-destructive examination of living cells in whole animals. First, our data indicate that the autofluorescent lipofuscin granules in the intestinal cells can be loaded with macromolecules which enter the cells by endocytosis. Using fluorescent labels, we found that both proteins (serum albumin, immunoglobulin) and dextran were sequestered in precisely the same granules which showed the blue autofluorescence characteristic of lipofuscin. Even after several days of observation, these macromolecules (or at least the fluorescent moieties) remained within the lipofuscin granules, showing that these were the final subcellular destination rather than an intermediate compartment.

Second, we have used the lysosomotropic agent acridine orange to show that the lipofuscin granules have an acidic internal milieu. The accumulation of acridine orange and the consequent red shift of fluorescence emission is generally taken as a diagnostic of the lysosome compartment [17,18,23]. The only other cellular organelles which are equivalently acidic [24] are the primary endocytic vesicles (so-called "endosomes"), but materials taken up by endocytosis pass rapidly through this compartment. Thus, the long-term occupation of the lipofuscin granules by fluorescent macromolecules shows that the granules are not primary endocytic vesicles.

Are the lipofuscin granules "telolysosomes"?

It has been frequently proposed [25,26] that lipofuscin granules contain a relatively large amount of indigestible or slowly digestible material and should therefore be identified with "residual bodies" or "telolysosomes". Our results demonstrate that the lipofuscin granules of *C. elegans* are active participants in the membrane-fusion cycle which brings exogenous materials into the lysosome compartment. Furthermore, the size distribution of the blue autofluorescent granules does not suggest that they are preferentially engorged with indigestible contents, although they can be made to swell upon uptake of a large dose of indigestible dextran (c.f. Fig. 3 to Fig. 2).

Nevertheless, it is not clear whether the lipofuscin-laden secondary lysosomes of older animals remain fully competent to digest the lysosomal contents. In *C. elegans*, the total content of many lysosomal acid glycosidases and phosphatases increases dramatically with age [14], although lysosomal cathepsin D reaches maximal activity in early adulthood and declines thereafter (L. Jacobson, unpublished observations). There is no evidence to indicate that these increased enzyme levels, measured by *in vitro* assays, reflect a similarly increased hydrolytic activity in the lysosome compartment *in vivo*. Thus, we do not know whether lipofuscin accumulation compromises the function of the secondary lysosomes in digesting materials of intracellular or extracellular origin.

The cellular sources of lysosomal lipofuscins

The localization of lipofuscins in secondary lysosomes in the intestinal cells raises the question of whether lipofuscins are originally formed in the lysosomes, or arise elsewhere and accumulate in the lysosomal compartment as a result of autophagy and/or membrane exchange. Isolated lysosomes can undergo oxidation *in vitro* to produce material with fluorescence excitation and emission spectra similar to those of lipofuscins [27–29], but there is little direct evidence that this is relevant to the mechanism of lipofuscin formation *in vivo*.

Regardless of where the lipofuscins are originally formed, it is potentially important that lipofuscin granules are active participants in endocytosis. If formation is principally intralysosomal, the uptake of exogenous materials by endocytosis would presumably provide the reactants for lipofuscin synthesis. If lipofuscins originate outside the lysosomes, endocytosis and/or autophagy might simply deliver preformed lipofuscins to the secondary lysosomes.

Using epifluorescence microscopy, we have detected no pronounced lipofuscin accumulation in *C. elegans* outside the intestinal cells, or in subcellular compartments other than the secondary lysosomes. In particular, we saw no indication of lipofuscin accumulation in mitochondria [which we identified by accumulation of the free fluorescent dye rhodamine 123 (cf. ref. 30)], or at the plasma membrane. It is entirely possible, however, that lipofuscins are initially formed in various subcellular compartments at levels too small to be detected by direct examination, and that only prolonged accumulation in lysosomes produces high enough concentrations for the granular autofluorescence to become easily visible. We hope to resolve this dilemma, at least for *C. elegans*, by using mutants deficient in endocytosis. Observations in our laboratory (G. Clokey and L. Jacobson, unpublished) indicate that in such mutants the punctate blue autofluorescence is greatly reduced relative to that in wild-type animals. It remains to be seen whether this reflects a lower cellular content of lipofuscins, or only a failure of these mutants to sequester lipofuscins in the secondary lysosomes.

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