Age-related changes in neutral lipid content of *Paramecium primaurelia* as revealed by nile red

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Abstract Cellular neutral lipid content of *Paramecium primaurelia* was measured during culture and clonal life using nile red (9-diethylamino-5H-benzo[α] phenoxazine-5-one), a dye utilized for lipid analysis in both mammalian cells and in ciliated protozoa. Lipid droplets in *P. primaurelia* are concentrated in the anterior pole of the cell; their number is maximum in early log phase cells and decreases in late log phase cells. The quantitative determination of neutral lipids was obtained measuring the fluorescence from the excitation and emission spectra of 480 nm and 540 nm, respectively. Neutral lipid content decreases linearly during the log phase of growth while the decline is minimum during the stationary phase. In the late log phase, the amount is 30% of that of the early log-phase cells. Though the cell size declines too, cell area and lipid content decreases are not correlated in the middle log phase, because the maximum lipid reduction is obtained when the cell size is relatively constant. The cellular lipid content also changes during the clonal life. Neutral lipids decrease discontinuously (r = -0.75, $P < 0.05$) as the fission age increases. No relationship was found between lipid content and food vacuole formation during both culture and clonal life.—**Ramoino, P., E. Margallo, and G. Nicolò.** Age-related changes in neutral lipid content of *Paramecium primaurelia* as revealed by nile red. *J. Lipid Res.* 1996. **37:** 1207–1212.

Supplementary key words culture growth • clonal life • lipid droplets • food ingestion capacity • fluorescence microscopy • ciliated protozoa

Nile red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, is an excellent vital dye that fluoresces intensely and whose intensity distribution does not vary in living or in fixed cells. The stained cells can be viewed for both yellow-gold fluorescence (excitation, 450–500 nm; emission, >528 nm) and red fluorescence (excitation, 515–560 nm; emission, >590 nm). Viewed for yellow-gold fluorescence, mammalian cells exhibit numerous small intensely stained bodies distributed throughout the cytoplasm; viewed for red fluorescence, cells reveal the same structures, but the individual bodies are not as readily resolved and, in addition, a diffuse general staining of the cytoplasm becomes more apparent (1). The intracellular lipid droplets are neutral lipids, usually triacylglycerols or cholesteryl esters: the former serve as fatty acid energy reserves and the latter function as storage depots for excess cellular cholesterol (2, 3). Nile red staining loses its selectivity for lipid droplets when viewed at fluorescence emission wavelengths >590 nm. Under these spectral conditions, nile red can be considered a general stain for lipids as it can interact and fluoresce in the presence of phospholipids, cholesterol, cholesteryl esters, and triacylglycerols (4). At fluorescence emission wavelengths >620 nm, nile red also fluoresces in the presence of fatty acid-free albumin, indicating that proteins containing hydrophobic domains, in addition to lipids, can induce nile red fluorescence (1).

The validity of using nile red fluorescence microscopy to obtain qualitative measures of lipid content, particularly cellular lipid droplets, has been documentated in mammalian cells (1, 5–8) and the validity of using nile red fluorometry to quantitate lipids has been shown in ciliated protozoa (*Paramecium caudatum, P. multimicronucleatum, P. tetraurelia,* and *Tetrahymena thermophila*) by the close correlation of nile red results (9) with lipid data obtained with gravimetric analysis (10). Therefore, nile red staining can be particularly useful when it is difficult to obtain the large number of cells required for gravimetric analysis.

In the present work nile red was used to study the pattern of neutral lipid accumulation in *P. primaurelia* during clonal life, i.e., in cells grown in excess of food, compared with the neutral lipid variation pattern observed during culture growth, i.e., in cells allowed to starve. The relation between lipid content and food uptake capacity of the cells is also investigated here.

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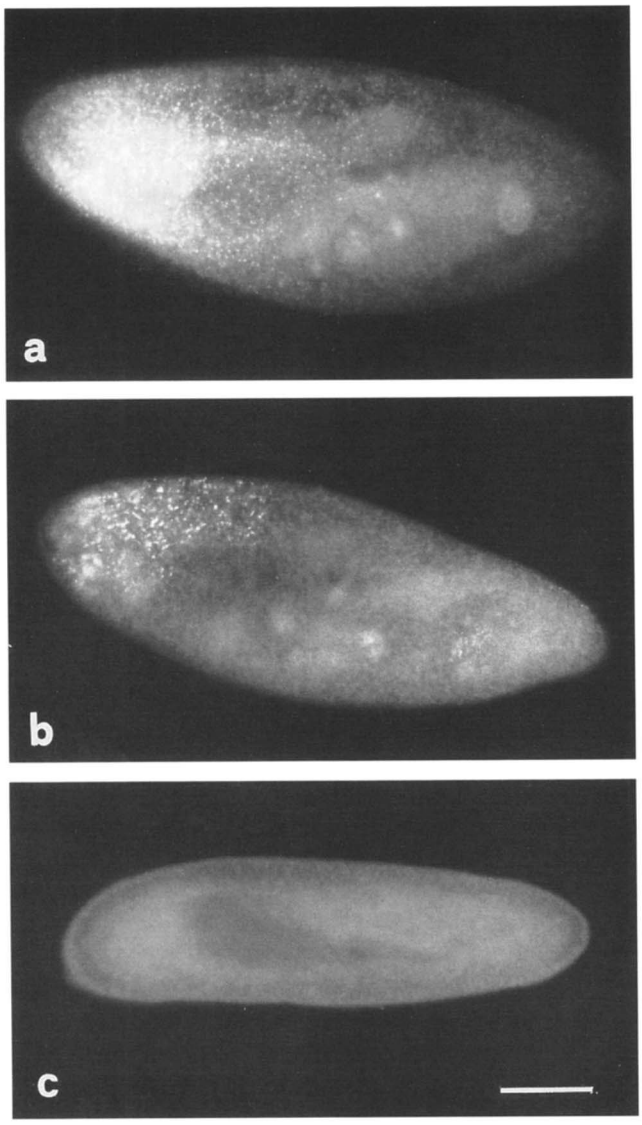

Fig. 1. Nile red fluorescence of cells of different culture ages: (a) day 1, (b) day 3, all in log phase of growth; (c) stationary phase (day 6). Bar, 20 μm.

MATERIALS AND METHODS

Material and culture methods

Cells of *Paramecium primaurelia*, stock 90, were grown at 25°C in lettuce infusion buffered at pH 6.9 and inoculated with *Enterobacter aerogenes* as monoxenic source of food. Conjugation was induced as described by Sonneborn (11) and exconjugant cells were separated thus giving rise to the experimental clones and transferred to depression slides containing fresh bacterized medium. Clones were cultivated according to two different methods in order to study the changes occurring through culture and clonal life.

Culture life. Clones were allowed to multiply in depression slides without any cell reisolation. On the 3rd day, when about 30–50 cells were present, clonal lines were transferred into test-tubes containing 15 ml fresh bacterized medium (day 0). No food was added to test-tubes during the culture growth, and food depletion occurred on day 5. Cell density of cultures was monitored daily to determine the phase of growth of the clones; density was estimated by counting live cells from at least five aliquots of a cell suspension. In our experimental conditions, the growth cycle of *P. primaurelia* consisted of log (days 1–4), stationary (days 5–9), and death phases (beginning with day 10). From day 1 to day 5 after the inoculum into test-tubes, cells divided daily; the maximum density was reached on day 5, i.e., at the beginning of the stationary phase. Death phase began on day 10 and then the population declined exponentially.

At different ages (expressed in days), samples that were constituted of about 50 randomly selected cells were extracted from clones and tested for their lipid content and food ingestion capacity.

Clonal life. The clonal lines were grown by daily reisolation in a pre-fixed amount of fresh bacterized medium (0.5 ml). The number of cells per depression derived from a single cell isolated the previous day was determined daily, and a single cell from each depression was isolated into a fresh culture medium. The $\log_2$ of the number of cells derived from a single cell was the number of fissions per day; the sum of the number of daily fissions from the conjugation up to a given day, was the fission age of that cell on that day. It was thus possible to know daily the clonal age from the conjugation.

At succeeding levels of the clonal age, synchronized cells obtained by manual selection of dividing cells of the desired fission age were isolated into individual drops of fresh culture medium, then were allowed to divide and grow, and their daughter cells were tested,

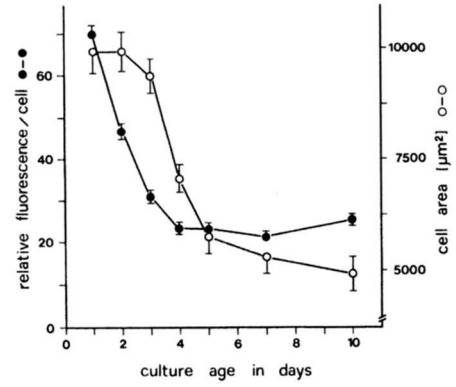

Fig. 2. Changes in neutral lipid contents and in cell size with culture age. Relative fluorescence values of neutral lipid content are expressed as fluorescent units. Vertical bars represent 95% confidence intervals of means. Sample size = 50–60 cells.

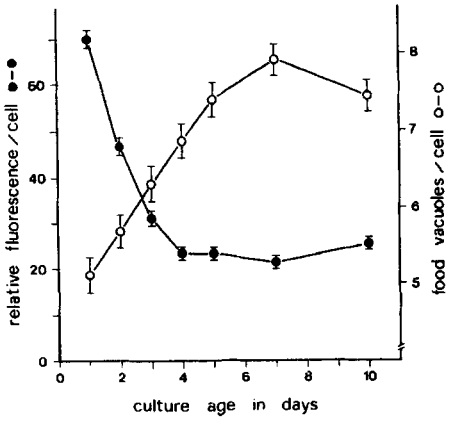

Fig. 3. Changes in neutral lipid content and in food vacuole formation with culture age. Relative fluorescence values of neutral lipid content are expressed as fluorescent units. Vertical bars represent 95% confidence intervals of means. Sample size = 50–60 cells.

for their lipid content and food ingestion capacity, 1 h after the fission (stage G_1 of cell cycle).

Samples of cells were routinely submitted to Dippell's cytologic test (12), in order to detect the occurrence of macronuclear fragments characterizing the autogamic process. Daily reisolation and tests were carried out up to the clonal decline which was signaled by the decrease in vitality and fission rate, by division asynchrony and monstrosity (morphologically abnormal paramecia, included chain of ciliates and unequal dividing cells).

Food vacuole formation

Food vacuole formation capacity was tested by staining the food vacuoles formed in 10 min with 0.02% latex particles (0.3 μm diameter). Cells were fixed with 2% glutaraldehyde and the labeled food vacuoles were counted. External environmental conditions were maintained constant throughout the experiments.

Cell size determination

The cell area was measured by means of a graphic tablet connected to an IBM computer.

Lipid staining and cytofluorometry

Cells of different culture and clonal ages were fixed with 5% formaldehyde in phosphate buffer (0.05 M, pH 7.4) for 30 min at room temperature, washed with phosphate buffer, stained with nile red (5 μg/ml, final concentration) for 30 min, and washed (9). The cells were observed with a Leitz microscope equipped with epifluorescence illumination, and pictures were taken with Kodak Tri X Pan 400 ASA film.

Nile red fluorescence was measured using a microphotometer MPV II Leitz equipped with epifluorescence optics. A photomultiplier S20 type 9558 AQ measured fluorescence intensities, and the values, expressed in fluorescence units, were read on a digital voltmeter. The excitation and emission wavelengths of 480 nm and 540 nm, respectively, were used to evidence the yellow-gold fluorescence emitted from neutral lipids (1, 9).

The controls for autofluorescence were cells that had not been exposed to nile red.

RESULTS

Culture life

When *Paramecium primaurelia* cells are stained with nile red and observed under a fluorescent microscope, lipid droplets fluoresce yellow-gold in a diffuse general staining of the cytoplasm. This diffuse staining is probably due to the labeling of the membrane lipids; indeed, some neutral lipids, such as triglycerides, function in the storage of lipids while others, especially steroids, are integral components of biomembranes (13, 14). Lipid droplets are concentrated at the anterior pole (**Figs. 1a, 1b**). Their number is maximum in cells of early log phase (day 1) and decreases throughout the log phase. By the stationary phase no droplets are seen (Fig. 1c).

The quantitative measure shows that the neutral lipid content per cell decreases linearly during the log phase of growth, whereas the decrease is minimum during the stationary phase (**Fig. 2**). The content in cells at the beginning of the stationary phase is 30% of early log phase cells. During culture life cell size also decreases (Fig. 2). Cell size changes can be accounted for, in part, by the different lipid content of cells; however, the cell size changes alone cannot completely explain lipid content variation as lipid content decreases when the cell sizes are relatively constant (days 1–3). Furthermore, the lipid content decrease is not linked to the decrease of food ingestion capacity, as food vacuole formation rate increases during log phase (**Fig. 3**).

No increase in lipid droplets is observed in day-1 cells compared to cells inoculated in fresh culture medium (day 0). Quantitative measures are not carried out because of the small number of cells available on day 0.

Clonal life

During clonal life tests were performed between 9 and 46 fissions, when clonal decline, evidenced by fission rate decrease and cellular anomaly appearance, began.

In young well-fed cells, lipid droplets are numerous and concentrated in the anterior pole of the cell; they decrease during clonal life, until they disappear at 31 fissions, whereupon they reappear briefly and decline again (**Fig. 4**).

The decrease of neutral lipid content during clonal life is lower than the decline observed during culture growth (**Fig. 5**). The neutral lipid amount in 46-fission cells is 66% of that of 9-fission cells. The neutral lipid decrease is not constant, and the linear regression analysis of data reveals a correlation coefficient $r = -0.75$, indicating that the neutral lipid diminution is related to the clonal age increase ($P < 0.05$). The highest and the lowest values are observed at 9 and 31 fissions, respectively, and the content in 31-fission cells is 61% of that of 9-fission cells.

Also, in cells grown in an excess of food, as already observed during the culture life (Fig. 3), the lipid content reduction is not related to a depressed food ingestion capacity, as the lipid content per cell decreases in early clonal life (9 to 15 and 28 fissions) when the food vacuole formation rate increases (Fig. 5).

DISCUSSION

Cellular neutral lipid content decreases during the culture life of bacterized *Paramecium primaurelia*, in agreement with data obtained for axenically grown *P. tetraurelia* and *P. multimicronucleatum* by using both gravimetric methods (10, 15–17) and nile red dye (9). As observed for *P. tetraurelia*, in *P. primaurelia* lipid droplets are concentrated at the anterior pole of the cell whereas in *P. caudatum* and *P. multimicronucleatum*, the droplets appeared to be evenly distributed throughout the cytoplasm. In *P. caudatum* and *P. multimicronuleatum* the number of lipid droplets is very high in cells of early culture life, so that the yellow-gold fluorescence covers the entire cells; by late log phase, the cellular lipid content is sufficiently reduced to allow individual droplets to be seen, and in the stationary phase, only few

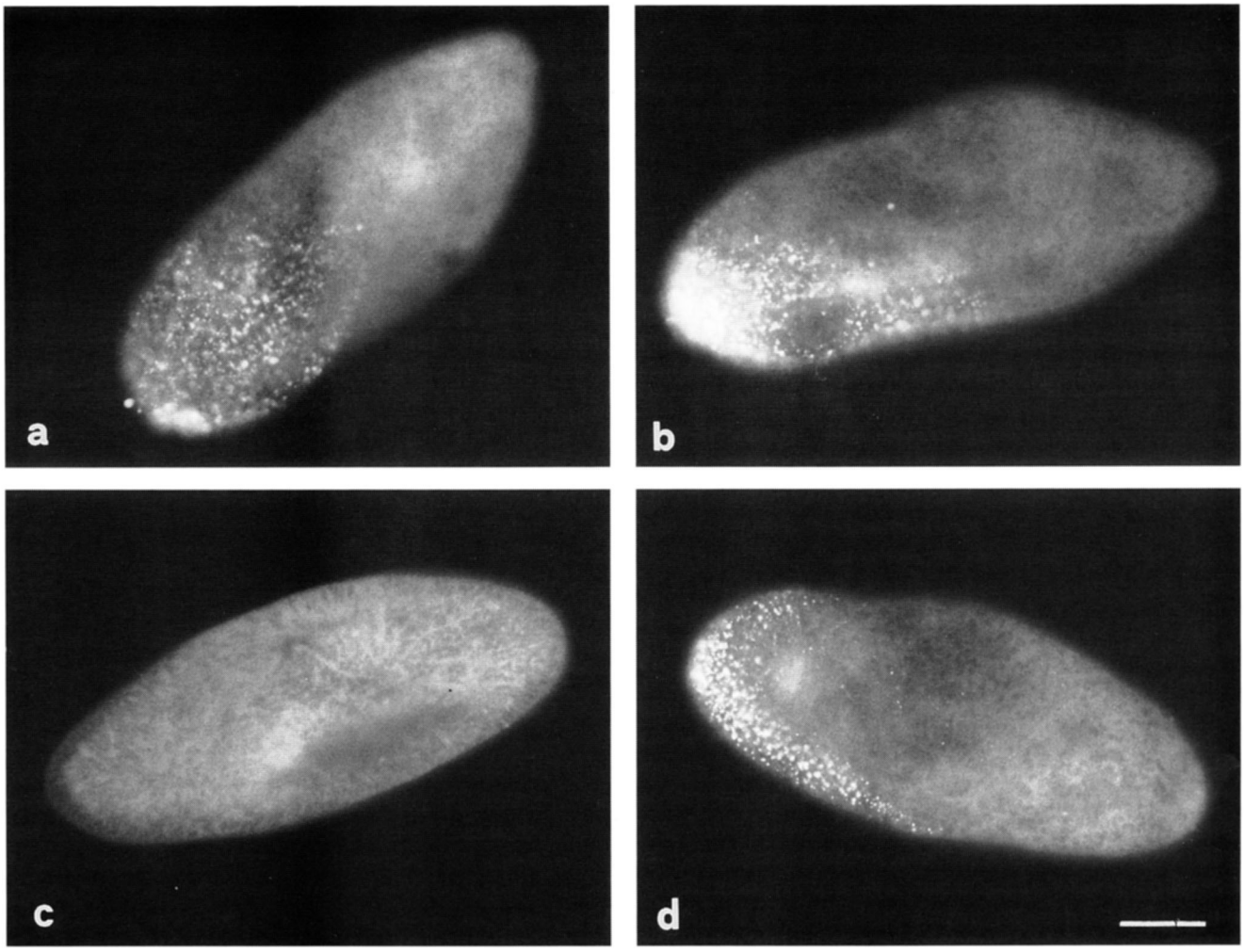

Fig. 4. Nile red fluorescence of cells of different clonal ages: (a) 9 fissions, (b) 24 fissions, (c) 31 fissions, and (d) 43 fissions; 46-fission cells show the same fluorescence pattern of 31-fission cells. Bar, 20 μm.

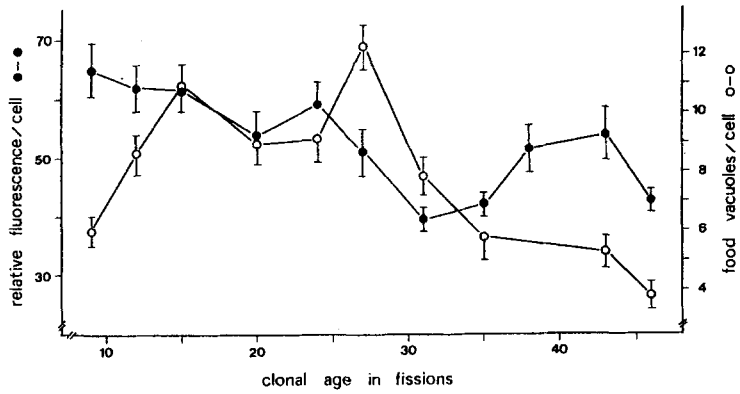

Fig. 5. Changes in neutral lipid content and in food vacuole formation with clonal age. Relative fluorescence values of neutral lipid content are expressed as fluorescent units. Vertical bars represent 95% confidence intervals of means. Sample size = 50–60 cells.

droplets are scattered in the cytoplasm (9). An opposite trend in the pattern of change in cellular lipid droplets with culture age was found in *Tetrahymena:* few lipid droplets were seen in young cells, whereas in stationary phase cells their number increased considerably (9). Indeed, *Tetrahymena* is capable of synthesizing its lipids (18, 19) whereas *Paramecium* needs to assume them from the medium (20). Exogenous lipids are accumulated into cytoplasmic vesicles and later metabolized and/or partitioned among daughter cells during log phase.

The changes in lipid contents reflect nutritional and metabolic conditions of the cell. Starved cells of axenically grown *P. caudatum* and *P. tetraurelia* show a 5-fold increase in cellular lipid content upon inoculation into fresh medium (10, 16). In the present study the increase upon inoculation was not observed because the cells inoculated were rejuvenated cells whose fission age was reset to zero. Indeed, *Paramecium* offers the advantage of reinitiating a new life cycle by conjugation or autogamy (21).

In axenically grown *P. caudatum,* lipid content in the medium is depressed with the culture cell density (22), and cultures enter stationary phase after lipid depletion in the medium, even when large amounts of proteins are still present (10). Therefore, it can be assumed that both in axenically and in bacterized grown *Paramecium,* the low lipid contents in stationary phase cells are due to the total depletion of lipids or bacteria in the medium, and that the cellular lipid content decrease in log phase cells is linked to a diminution of food available per cell. Indeed, cultures are not added with fresh culture medium after their inoculum into test-tubes and cell density increases during log phase because of cellular division.

No data are available regarding changes in lipid content during clonal life of *Paramecium.* This work gives the first evidence that neutral lipid content, revealed by nile red, decreases also in cells reisolated daily in excess food. The decrease is not only not due to the depressed food available, but is also not linked to a diminution of food ingestion capacity as food vacuole formation rate increases in early clonal life. In paramecia grown in bacterized medium, food vacuole formation is the major mechanism of food ingestion: bacteria and particle material enter the cell via the cytopharyngeal area where food vacuoles are formed (23). Conversely, in axenically grown *P. tetraurelia,* fatty acids are taken up by carrier-mediated transport, not by bulk transport and simple diffusion (24, 25). Cells release lipases into the medium that can degrade complex lipids (reported in ref. 24) and the products such as fatty acids thus released can then be readily taken up as free fatty acids.

The decrease of neutral lipid content during clonal life could be due to a decreased capacity of digestion of food taken up. Indeed, lysosomal enzyme activity varies with fission age in cells continuously supplied with an excess of food; in particular, the content of the acid phosphatase (an enzyme marker of lysosomal activity) decreases in the early clonal life when the food ingestion capacity increases (26). The insufficient amount of lysosomal enzymes could lead to a slower digestion, to the possibility of egestion of non-completely digested material, and to a lower lipid droplet number. The increase in neutral lipid content at 35 to 43 fissions could be linked to an increased lysosomal enzyme activity. In effect, an increase in acid phosphatase content follows the previous decrease exhibited during the early clonal life (26).

In summary, neutral lipid content in *P. primaurelia* decreases both during the log phase of the culture growth and during the clonal life, even when the reduction is lower during the latter stage. The fission age increase leads, in young well-fed cells (clonal life), to a lipid decrease of about 40% whereas it leads, when connected with food reduction (log phase of culture

life), to a diminution of 70%. Therefore, in addition to the food depletion, it is necessary to take into account the age of the cells to obtain consistent and reproducible data.

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