LOSS OF ENDOCYTIC CAPACITY IN AGING *PARAMECIUM*

The Importance of Cytoplasmic Organelles

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ABSTRACT

Aged cells have significantly fewer food vacuoles and ingest fewer bacteria than young cells. Loss of food vacuoles was explained by a decreasing difference in the food vacuole formation and excretion rates; the formation rate declined more rapidly than the excretion rate, approaching equivalence at 160 fissions, when the proportion of cells with no food vacuoles, in the presence of excess food, abruptly increased. A model for cellular aging is presented in which control of organelle numbers and cyclical interactions between the nucleus and cytoplasm may be of critical importance.

The classical studies of Sonneborn (40) showed that paramecia age and die unless they undergo the rejuvenation processes of either autogamy (self-fertilization) or conjugation (cross-fertilization). The life cycle of vegetatively dividing paramecia is characterized by a developmental sequence during which, subsequent to the sexual process, cells undergo a predictable average number of vegetative divisions (150–200 fissions), passing seriatim from a period of immaturity (when the cells cannot mate or undergo autogamy), to maturity (when the cells can mate or undergo autogamy), to senescence (when there is a decreased probability that a given cell will give rise to a viable cell at the next division), and finally to cell death (40, 33). Studies have also shown that normal human cells in tissue culture grow old and die (12, 15); many similarities have been noted between human cells in culture and aging paramecia (14).

The process of intracellular digestion in paramecia is characteristic of the process of intracellular digestion found in most eukaryotic cells (7, 26). In general, the process begins when exogenous particles or solutes, be they nutritive or nonnutritive, are internalized within the cell by means of an endocytic vacuole, be it of the phagocytic or pinocytic variety. In paramecia, vacuoles are formed from a specialized region at the base of the oral apparatus, which undergoes rapid membrane expansion and invagination to be subsequently pinched off as a food vacuole (1, 17). The vacuoles then circulate in the cytoplasm where digestion of the intravacuolar contents occurs. All undigested material is excreted from the cell by exocytosis, during which the food vacuole membrane fuses with the plasma membrane and is recycled back to the oral apparatus by a network specially designed for membrane recycling (1, 2).

In the present study, changes in endocytic activity (the rate of formation and excretion of food vacuoles and number of bacteria ingested) were monitored as a function of cellular age. The assumption was made that the number of food vacuoles per cell is determined primarily by the relative rates of formation and excretion of food vacuoles. For example, if a newly divided cell contains 10 food vacuoles and if during its interfission period it forms 200 and excretes 190, the difference (200 − 190 = 10) of 10 represents the number of food

vacuoles added during the interfission period to the initial pool of 10 vacuoles. The cell would thus have a total of 20 food vacuoles immediately before division to provide an average of 10 vacuoles for each of the two daughter cells. Had the initial number been other than 10, and the relative rates the same, the average value of 10 would be approached rather quickly with successive fissions. Age-correlated changes in either the formation or excretion rates would be expected to be reflected in the average total number of food vacuoles per cell. Therefore, the average number of food vacuoles, the rates of formation and excretion of food vacuoles, and the net increase in food vacuoles during the interfission period (the difference in the number of food vacuoles formed and excreted) was determined as a function of increased cellular age. The data represent the first evidence for loss of regulation of organelle numbers and reduced bacterial ingestion with advanced clonal age, and also provide a model for dilution of organelles and/or unit substances as cells undergo life cycle changes under constant external environmental conditions.

MATERIALS AND METHODS

Culture Conditions and Techniques

Standard Culture Conditions

Stock Cultures: cells of *Paramecium tetraurelia,* stock 51, mating type VII, were used. These have been kindly supplied by Dr. T. M. Sonneborn, Indiana University; these cells were used since autogamy can be induced by starvation when the cells are about 20 fissions old, and autogamy rarely occurs in daily isolation lines grown under favorable conditions (40). The culture medium was Cerophyl medium inoculated 24 h before use with *Klebsiella aerogenes,* incubated at 27°C, and adjusted to pH 6.7 immediately before use.

Maintenance of Aging Cell Lines

Our selective procedures differ from those of Sonneborn (40) and are described below. Single cells were maintained in plastic disposable spot plates (96 depressions per plate). Autogamy indicates zero time, and was routinely ascertained by acridine orange (0.3 mg/ml) staining and fluorescence microscopy. This staining procedure allows visualization of the nuclear changes typical of autogamy (35). When 100% of an observed sample isolated from a given depression exhibited autogamy, eight unstained sister cells were removed from that depression with a micropipette under a dissecting microscope, and each one was transferred as single isolates to a depression containing fresh food. The following day, the number of cells per depression derived from a single cell was determined; a single cell from each depression was reisolated and given fresh food. Competent cells not transferred were permitted to undergo autogamy and served as the source for initiating new progeny lines. The procedure of counting cells and reisolation of cells was repeated daily. The $\log_2$ of the number of cells derived from a single cell is the daily fission rate. The number of fissions per day divided into 24 h represents an estimate of the time taken for one interfission period. The sum of the number of daily fissions from the date of origin to a given day is the fission age of the cell. The total number of fissions from the origin of the clone to the death of an isolated cell is the life span of that cell line. The average life span of the clone is the mean of the life span of the eight sublines in the isolation line. If a subline undergoes autogamy in the isolation line, it is dropped from the determination of the cell life span because it will start a new generation.

Number of Food Vacuoles Per Cell

The procedures of Lee (21) and Preer (29) were used to estimate the number of vitally stained vacuoles. The cells were maintained at 27°C and the pH was adjusted to 6.7, since the temperature and the pH of the medium have been shown to affect the number of food vacuoles formed (21, 22). Samples of about 160 cells of the desired fission age were obtained from daily isolation lines and allowed to grow for 24 h in excess culture fluid in standard plastic petri dishes to obtain mass cultures. A dilute suspension of India ink was then added to the culture to facilitate visualization of the food vacuoles. The fission age increase of the cells in mass culture was estimated from the increase in age of sister cells in the daily isolation lines for the same time interval (2–4.5 fissions). Samples of cells from mass cultures were assayed for correlation of observed fission rate with expected rate and stained for autogamy. Cultures which exhibited either a deviant fission rate or cells in autogamy were discarded. Since we wished to compare the mean number of food vacuoles in old and young cells, and since the interfission time is longer for older cells, a fair estimate could only be obtained using synchronized cells; mass cultures of young cells would contain more recently divided cells than mass cultures of old cells. Thus, dividing cells from the mass cultures were selected under a dissecting microscope to obtain synchronized cells. By sampling cells at hourly intervals, the number of food vacuoles was determined throughout the cells' interfission cycles (until the cells were observed to be in division again). The actual counts of food vacuoles were made by fixing samples of cells with 1:1 absolute ethanol:40% formaldehyde.[1] This fixative was found to best preserve the intact food vacuole when a cover slip is placed on a preparation of cells. The number of darkly

[1] Jenkins, R. A., University of Wyoming. Personal communication.

stained membrane-bounded bodies was then counted in a phase microscope.

Rate of Formation of Food Vacuoles

Food vacuoles were labeled with either a dilute suspension of India ink or carmine particles. Formation of vacuoles was observed over a 10- or 20-min interval. To determine the rate of formation of food vacuoles in living cells, a single cell was placed in India ink-food and inserted by capillary action into a microslide (rectangular capillary tubes which are 0.1 mm path length, Vitro Dynamic, Rockaway, N. J.), and formation of colored vacuoles was observed under phase microscopy for 10 min. This cell was then blown out of the capillary, rinsed into carmine-food, reinserted into the capillary, and observed for the formation of red vacuoles. Two colors were used since it was found that cells begin to excrete newly formed food vacuoles approximately 10 min after their formation. The total number of black vacuoles formed after 10 min was added to the number of new red vacuoles formed per unit time for the succeeding time intervals after 10 min. This procedure was found to minimize the influence of excretion on the apparent rate of food vacuole formation. The total number of vacuoles vs. time in minutes was plotted, and the slope of the regression line was determined as the rate of formation. Alternatively, cells were placed in one colored food for 10 min, the cells were fixed, and the total number of colored vacuoles was counted. The total number of vacuoles divided by 10 was the value for the rate of formation of food vacuoles per minute.

Rate of Excretion of Food Vacuoles

Cells were incubated overnight in culture fluid supplemented with India ink to color all of the food vacuoles. Samples of cells were removed and the average number of colored vacuoles was determined, while other cells were washed into uninoculated Cerophyl medium. The number of colored food vacuoles in cells at various intervals in uninoculated medium was determined. The difference in the mean number of colored vacuoles present and the mean number at a given time after the cells were placed in uninoculated medium was the mean loss of food vacuoles per unit time. The mean loss of food vacuoles divided by the time interval was the excretion rate of the food vacuoles.

Determination of the Difference Between Formation and Excretion Rates

The increase in the number of food vacuoles from the number found immediately after cell division to the number present before the next division was determined in cells with India ink-colored food vacuoles. The increase in the number of food vacuoles was divided by the interfission time to obtain the rate of increase in the number of food vacuoles per unit time. The rate of increase in food vacuoles must equal the rate of formation minus the rate of excretion. (If a cell forms 300 food vacuoles and excretes 300 food vacuoles, there can be no increase in the number of food vacuoles.) When the difference between formation and excretion rate is calculated using this procedure, a relatively accurate estimate of the difference in the formation and excretion rates can be obtained. This method is much simpler than attempting to determine both the formation and excretion rates throughout the cell cycle for many clones.

Determination of Particle Number Per Food Vacuole

VOLUME OF FOOD VACUOLES: The volume of food vacuoles was determined on newly formed food vacuoles since the size of the vacuoles is known to change after time in the cytoplasm (25). The unlabeled cells were placed in India ink for 3 min, fixed with 1:1 absolute ethanol:40% formaldehyde and observed without a cover slip in a phase microscope. The diameter of the food vacuole was estimated with an ocular grid. Observations of rotating food vacuoles in living cells indicate that food vacuoles are normally spheroid; therefore, the volume of the food vacuole was calculated using the formula $(4/3)\pi r^3$.

DENSITY OF BACTERIA PER FOOD VACUOLE: In order to determine the average bacterial density within food vacuoles in which digestion had not yet or just barely begun, electron microscopy of random food vacuole sections, in which there were intact bacteria and no evidence of bacterial ghosts, was carried out. Old and young cells were fixed for 90 min in 0.1 M collidine-buffered, 1% osmium tetroxide at pH 6.8, stained for 15 min in 0.5% uranyl acetate, dehydrated and embedded in n-butyl ethyl methacrylate at 60°C for 12 h. Sections were cut with a Porter-Blum ultramicrotome and viewed on an RCA EMU 3G electron microscope. Magnification, enlarger settings, and section thickness were kept constant. In calculations of the bacterial density, a grid was placed over an 8 × 10 photograph of the food vacuole section, and the number of square centimeters was counted. The number of bacteria per square centimeter was then determined, and this value was converted to the number of bacteria per 10^3 μm^2 in the intact organism (2 cm = 1 μm), thus providing the density of bacteria per section of food vacuole.

CONCENTRATION OF INDIA INK PARTICLES NEEDED TO LABEL CELLS: Dilutions of the stock India ink solution (Higgins Black, Faber-Castell Corp., Newark, N. J., no. 4415) were carried out in bacterized Cerophyl medium at pH 6.7. Young and old cells were then fed the various concentrations of India ink particles for an interval of 5 min, fixed, and observed under the low power objective of the phase microscope. The lowest titer necessary to label food vacuoles was determined as that titer at which no particles could be observed in newly formed food vacuoles.

NUMBER OF POLYSTYRENE BEADS INGESTED PER FOOD VACUOLE: Cells of different ages were incubated for varying lengths of time in the same concentration of washed, inert 0.8 μm polystyrene beads (Dow Chemical, Midland, Mich.) suspended in uninoculated Cerophyl medium (29). The cells were then fixed with 45% acetic acid, which was found to make the food vacuole membrane highly labile, squashed with a cover slip, and the number of beads per food vacuole was counted.

DIRECT COUNTS OF THE NUMBER OF BACTERIA PER FOOD VACUOLE: To estimate the number of bacteria directly, it was necessary to avoid vacuoles in the process of digestion. Therefore, cells were incubated for 3 min in a very dilute suspension of carmine particles in bacterized Cerophyl, stained with acridine orange (0.3 mg/ml), fixed with 45% acetic acid, and squashed. The newly formed vacuoles were identified by the vacuoles containing a few grains of carmine particles, and the bacterial counts were made by estimating the number of bacteria seen in each flattened and enlarged, still distinct food vacuole using a 100× Zeiss objective and an ocular grid.

RESULTS

Mean Number of Food Vacuoles

The mean number of food vacuoles in cells grown under constant external environmental conditions was determined as a function of cellular age. Fig. 1 represents the results of 16 experiments in which the mean number of food vacuoles was determined for cells of varying ages. Each point represents a minimum of 100 synchronized cells examined. An average population of young cells could be expected to have 22 food vacuoles per cell, and an average population of old cells, five food vacuoles per cell. Linear regression analysis of these data reveals a coefficient of correlation (r), equal to 0.92, indicating a significant nonzero relationship. The coefficient of determination, (r^2), equals 0.84, implying that increasing fission age "explains" 84% of the variation found in food vacuole numbers. This finding alone does not necessarily imply reduced capacity of vacuole formation in aged cells; an old cell could simply be forming and excreting the vacuoles at faster rates.

Food Vacuole Formation Rate

The data indicate that aged cells have a reduced rate of food vacuole formation (Table I, Figs. 2 and 3). Rates of formation of food vacuoles are depressed in old cells not only when randomly selected cells from mass cultures of young cells are compared with randomly selected cells from mass cultures of old cells (Table I), but also when the rate of formation of synchronized young cells is compared with the rate of formation of synchronized old cells (Fig. 2). Cells 25 fissions old can form an average of one food vacuole per minute, whereas cells 160 fissions old exhibit one-fifth that rate of formation (Table I). When synchronized cells were observed forming food vacuoles at hourly intervals after division, at no time did the rate of food vacuole formation in aged cells exceed the rate found in young cells (Figs. 2–3). The data also provide evidence that the rate of formation of food vacuoles increases during the interfission cycle (Fig. 3); this increase is found in both the young and old clones, even though the rate of formation of food vacuoles is reduced in the old clones.

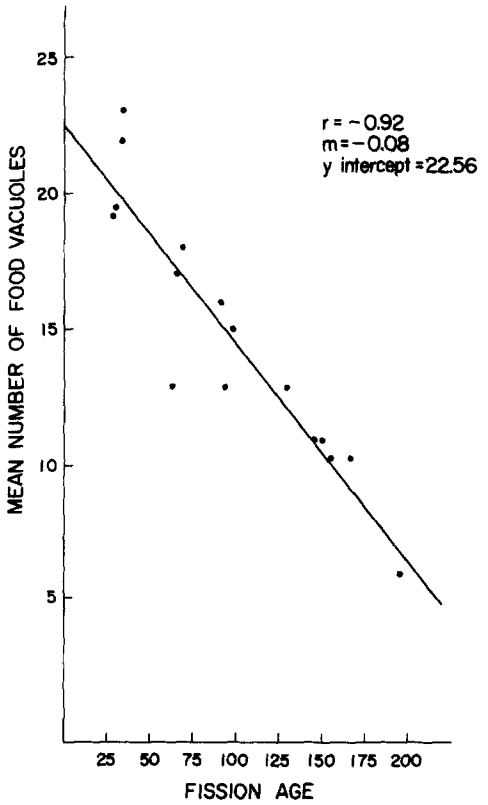

FIGURE 1 Mean number of food vacuoles with increased cellular age. The number of food vacuoles in cells grown under constant external environmental conditions was determined as a function of cellular age. The average number of food vacuoles in populations of young cells was 22 per cell, in populations of old cells, five per cell.

Total Number of Food Vacuoles Formed During the Interfission Interval

The total number of food vacuoles formed during the interfission interval was estimated for clones as clonal age increases to determine whether the increased interfission time for aged clones could compensate for the reduced rate of formation of food vacuoles. Data in Table II provide evidence that aged cells form significantly fewer food vacuoles during the interfission interval than young cells; clones 30 fissions old form an average of 282 food vacuoles, while clones 150 fissions old form only 225 food vacuoles.

TABLE I

Rate of Food Vacuole Formation

	Age (fissions)						
	25	45	65	85	115	130	160
Rate of food vacuole formation* (no. formed per min)	1.03	0.88	0.86	0.71	0.47	0.48	0.20

* Each value is a mean of 120 cells. The student's *t*-test was used to compare the differences observed. Those means not underlined by the same line are very significantly different from each other ($P < 0.05$).

Rate of Excretion of Food Vacuoles from Unsynchronized Cells

Fig. 4 shows a representative experiment indicating that the rate of excretion of food vacuoles was estimated using the linear portion of the

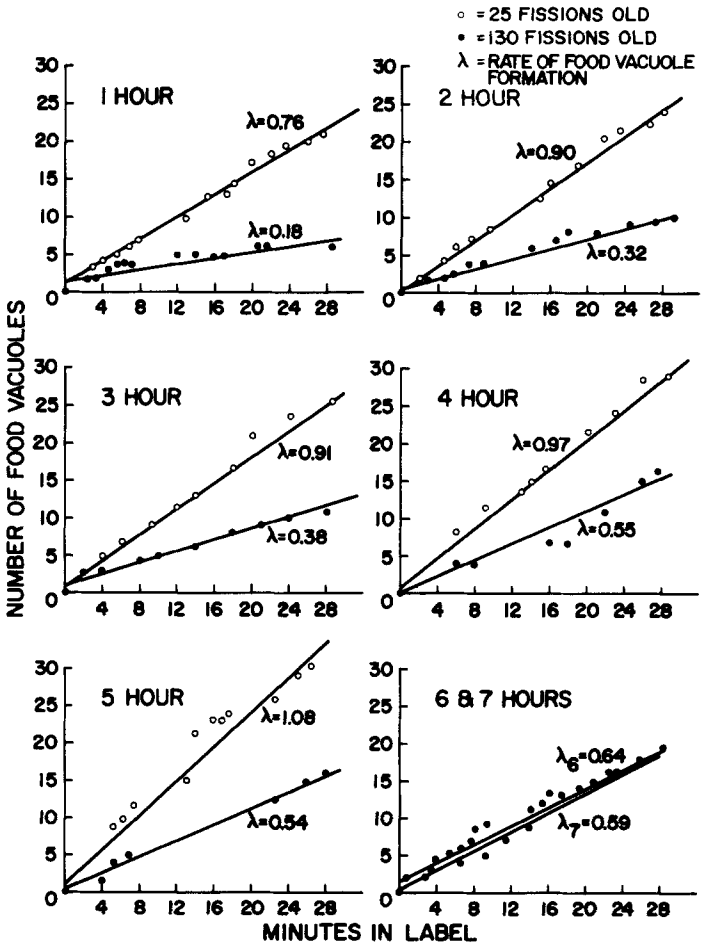

FIGURE 2 Rate of food vacuole formation in synchronized cells. Synchronized living cells were observed in rectangular capillary tubes forming first black (India ink) and then red (carmine) food vacuoles. The rate of formation of food vacuoles was always greater in the young than the old cells. The 6 and 7 h time points have only old cells since the young cells had already divided.

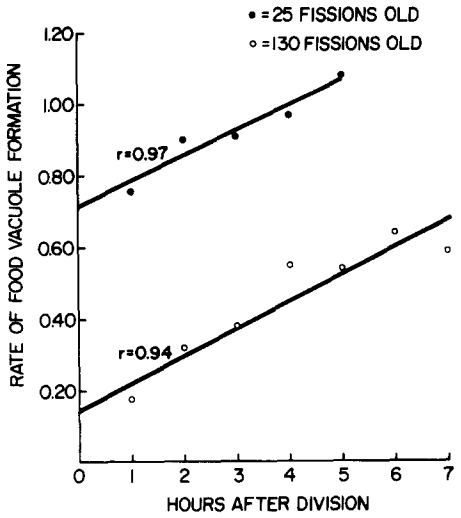

FIGURE 3 Increase in food vacuole formation during the interfission period. The rate of formation of food vacuoles seen in Fig. 2 was plotted vs. time after division. The resulting lines were subjected to linear regression analysis, and the data indicate a significant non-zero relationship in both the young and old clones; the coefficients of correlation were 0.97 and 0.94, respectively. The data were taken as evidence that the rate of formation of food vacuoles increases during the interfission interval.

TABLE II

Mean Total Number of Food Vacuoles Formed per Interfission Interval as Age Increases

	Age (fissions)				
	30	60	90	120	150
Food vacuole formation (FV/min)*	0.88	0.84	0.71	0.50	0.50
Interfission time (min)‡	320	325	350	410	450
Mean no. food vacuoles formed§	282	273	249	205	225

* The sample size was 60 cells for each age examined. FV = food vacuole.

‡ The interfission interval was calculated from the daily isolation line data (see Materials and Methods).

§ The mean number of food vacuoles was calculated by multiplying the food vacuole formation rate by the interfission time. Those means not underlined by the same line were found to be significantly different ($P < 0.05$) using the student's *t*-test.

curves, and was found to be 0.80, 0.70, and 0.17 food vacuoles excreted per minute to clones 45, 127, and 161 fissions old, respectively (Table III). The excretion rate was found to decrease significantly with increased clonal age.

Difference in the Number of Food Vacuoles Formed and Excreted

The ability of aging clones to maintain a constant increase in numbers of food vacuoles during the interfission period depends on a mean constant different in the total number of food vacuoles formed and excreted; e.g., if a cell has 10 food vacuoles, forms 200, and excretes 190, then the difference (200 − 190 = 10) represents an increase of 10 food vacuoles for a total of 20 before the next cell division. Estimates of the formation and excretion rates were made and the

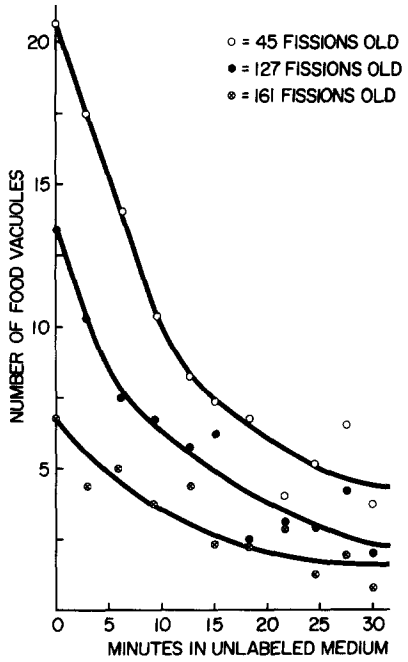

FIGURE 4 Rate of food vacuole excretion with increased cellular age. Cells were incubated overnight in bacterized Cerophyl with India ink to color all of the food vacuoles. The mean number of black vacuoles at zero time and at various intervals of time after the cells had been incubated in uncolored, uninoculated Cerophyl medium was determined. The decline in food vacuole number was exponential in both the young and old clones. Using the linear portion of the curve, the rate of excretion of food vacuoles after 20 min was calculated and converted to food vacuoles lost per minute (initial number of food vacuoles minus number of food vacuoles after 20 min/20). The rates of excretion for cells 45, 127, and 161 fissions old were 0.80, 0.77 and 0.17, respectively.

TABLE III

Difference in the Number of Food Vacuoles Formed and Excreted during the Interfission Interval in Aging Cells

Age (fissions)	Rate: Food vacuole Formation* (FV/min)§	Rate: Food vacuole Excretion‡ (FV/min)§	Interfission time (min)	Difference in no. FV formed minus no. FV excreted during interfission interval‖
45	0.84	0.80	330	277 − 264 = 13
127	0.78	0.77	340	265 − 262 = 3
161	0.17	0.17	450	77 − 77 = 0

* Two groups of 20 cells for each age were examined.

‡ Excretion rates were taken from Fig. 4.

§ The formation and excretion rates are expressed as the number of food vacuoles (FV) per minute.

‖ The total number of food vacuoles formed was estimated by multiplying the food vacuole formation rate by the interfission time (min). The total number of food vacuoles excreted was calculated by multiplying the excretion rate by the total interfission time (min). The difference between the total number of food vacuoles formed and excreted was then determined.

rates were used to calculate the total number of food vacuoles formed and excreted during the interfission interval. Table III represents the results of a typical experiment indicating a decline in the difference in the number of food vacuoles formed and excreted. Clones 45, 127, and 161 fissions old were found to increase their food vacuole number by 13, 3, and 0, respectively, during the interfission interval. Note that two variables contributed to the decline: (*a*) the total number of food vacuoles formed was reduced, and (*b*) there were relatively more food vacuoles excreted.

Since the difference in the number of food vacuoles formed and excreted should equal the number of food vacuoles increased during the interfission period, an independent and more accurate estimate of the difference in the rates of formation and excretion of food vacuoles could be made by directly determining the increase in food vacuoles in synchronized cells. The difference in the number of food vacuoles at predivision and postdivision (newly divided cells) should equal the increase in food vacuole number during the interfission interval as well as the difference in the number of food vacuoles formed and excreted. Table IV shows that clones 30 fissions old increase their food vacuole number by 16.6 during the interfission interval, whereas clones 150 fissions old increase their food vacuole number by only 6.4 food vacuoles. Note that the estimates of the increase in food vacuoles obtained by using the difference in formation and excretion rates (Table III) correlate well with the observed increases in food vacuoles seen in Table IV. The assumption that the difference in formation and excretion should equal the increase in food vacuole number therefore appears to be a valid assumption. If the rate of increase of food vacuoles per minute is multiplied by 100 and is used as an index to compare clones of increased age, the difference in the formation and excretion rates is shown to decline (Fig. 5). The correlation coefficient for these data is significantly non-zero at the $P = 0.02$ level, indicating a real decline in the difference between formation and excretion rates as age increases.

Fig. 6 illustrates the consequences of varying the decline in formation and excretion rates of food vacuoles with increased clonal age. Clearly, only when the rate of formation of vacuoles declines at a rate faster than the rate of excretion can the rate of increase in food vacuole numbers decline to a point where the rate of formation eventually equals the rate of excretion (as seen in Tables III and IV). When the two rates (excretion and formation) become equal, there can be no increase in food vacuole number, and, if the decline in formation rate continues, the excretion rate will exceed the formation rate, rapidly leading to cells with no food vacuoles.

The above data imply that the inability of cells to increase food vacuole number during the interfission cycle with increased clonal age is a result of a faster deterioration in the formation rate than the excretion rate of food vacuoles. This would be expected to lead to both a gradual decline in food vacuole numbers and a rising frequency of loss of

TABLE IV

Increase in Food Vacuole Number during the Interfission Cycle as Clonal Age Increases

	Age (fissions)				
	30	60	90	150	196
Mean no. of food vacuoles*					
Pre-division	29.8	20.9	17.2	12.6	5.8
Post-division	13.2	9.7	10.4	6.2	4.1
Increase in FV no.‡	16.6	11.2	6.8	6.4	1.7
Interfission interval§	285	340	360	390	630
Rate of increase in FV/100 min‖	5.8	3.3	1.9	1.6	0.3

* The sample size was at least 120 cells for each age (four groups of 30 cells).

‡ The difference in the mean number of food vacuoles at predivision and postdivision represents the increase in food vacuoles during the interfission cycle. Those means not underlined by the same line are significantly different from one another using the student's *t*-test.

§ The interfission time was the observed interval of time from one cell division to the next (in minutes).

‖ The increase in food vacuole number during the interfission interval was divided by the interfission time (min) to obtain the rate of increase in food vacuole number in food vacuoles per minute. This value was multiplied by 100 to emphasize the effect over a longer time interval. Linear regression analysis of the rate of increase reveals a very significant non-zero relationship ($P = 0.02$) indicating a real decline with age.

all food vacuoles. The gradual loss is illustrated as follows: even if an aged cell were to have as many food vacuoles at the beginning of the interfission cycle as a young cell (viz. 13), its depressed ability to increase food vacuoles (viz. 6 food vacuoles) could only provide the cell with a total of 19 vacuoles immediately before the next division, and, upon division, the daughters would contain a mean of only 9.5 food vacuoles, or a net loss of 3.5 food vacuoles per cell (13 − 9.5). As can be seen by this example, the most important variable is the ability to maintain a net constant increase in food vacuole number during the interfission cycle; this ability continuously declines as clones age, resulting in the observed gradual reduction in food vacuole numbers. When the rate of excretion exceeds the rate of formation, an abrupt appearance of cells with no food vacuoles would also be expected. Table V shows that the number of cells with no food vacuoles abruptly increases at an age when the difference in the formation and excretion rates is very small. Intraclonal variation in formation and excretion rates would be expected to produce some cells which have a rate of excretion greater than the formation rate, thus providing an explanation for the cells with no food vacuoles. Chance distribution at fission could also account for a substantial amount of intraclonal variation, particularly when the number of food vacuoles becomes small.

Number of Particles Ingested

The observation that old cells form fewer total food vacuoles during the interfission period does not necessarily imply that there is less ingestion of bacteria. The possibility that aged clones form larger food vacuoles, and thus accommodate more bacteria per food vacuole, was investigated. Table VI shows that the volume of newly formed food vacuoles is not significantly different as clonal age increases, though aged cells tend to have smaller food vacuoles. In order to examine the possibility that aged cells have a higher density of bacteria per food vacuole, electron microscopy of newly formed food vacuoles (vacuoles in which the bacteria were observed to be morphologically intact) was carried out in young and old clones. Upon determination of the number of bacteria per 10^3 μm^2, cells 38 fissions old were found to contain an average of 184 bacteria and cells 155 fissions old, 162 bacteria. Food vacuoles from 5 old cells and 10 young cells were compared, and the reduction in bacterial density was significant at the 0.05 level using the Mann Whitney U statistic (37). When considered together, the observation that aged cells do not have larger food vacuolar volume and exhibit a reduced density of bacteria, the data lend supportive evidence that aged cells ingest fewer bacteria per food vacuole.

Though the findings described above imply that

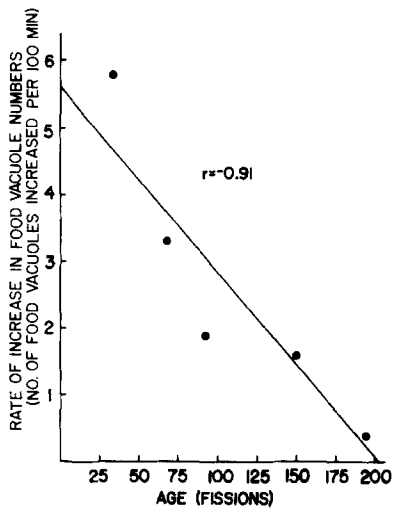

FIGURE 5 Difference in formation and excretion rates as a function of clonal age. The difference in formation and excretion rates was determined as follows: the increase in food vacuole number during the interfission interval was observed as described in Table IV (by the increase in the number of food vacuoles immediately after division to immediately prior to the next division). The rate of increase in food vacuoles per minute equals the number of food vacuoles increased divided by the interfission time (in minutes). Since the rate of increase can be assumed to equal the formation rate minus the excretion rate, the rate of increase is an estimate of the difference between the two rates. The data provide evidence that the difference in the rates of formation and excretion declines with advanced clonal age. The coefficient of correlation represents a non-zero relationship significant at the $P = 0.02$ level.

old cells concentrate fewer bacteria into their food vacuoles than young cells, this was verified using three different approaches: (*a*) The concentration of India ink particles necessary to label food vacuoles was found to be dependent upon clonal age. At a dilution of 1:1,500 of the stock India ink solution in bacterized Cerophyl medium, all of the food vacuoles of young cells are clearly colored black when viewed under low power of the phase microscope, whereas none of the food vacuoles in old cells are discernible at this concentration of black particles. (*b*) Ingestion of latex beads per food vacuole was found to be dependent upon clonal age. Table VII shows a comparison of the number of beads internalized per food vacuole as clonal age increases. The data reveal a significant reduction in number of latex beads incorporated per food vacuole with increasing clonal age. Cells 7 fissions old can concentrate an average of 480 beads per food vacuole, whereas cells 162 fissions old can only concentrate an average of 143 beads per food vacuole under the same external environmental conditions. (*c*) Ingestion of bacteria per food vacuole was found to be dependent upon clonal age. Direct counts of the number of bacteria per food vacuole were made with squashed preparations of cells in the phase microscope. Variability in bacterial counts between experiments was found. At least one important variable in the determination of number of bacteria per food vacuole was the concentration of bacteria in the medium. When the concentration of bacteria was varied, increased bacterial concentration resulted in increased numbers of bacteria per food vacuole in both the young and old cells. Our results further confirm the observations of Preer (29) that the number of particles ingested per food vacuole is a function of particle concentration, but that the rate of formation of food vacuoles is not dependent upon the titer of particles present in the medium. These relationships are maintained in aging cells. Nevertheless, in all four comparisons made between young and old cells under constant external environmental conditions, the young cells ingested significantly more bacteria per food vacuole than aged cells (Table VIII). Since aged cells form less total food vacuoles per interfission cycle, and incorporated less bacteria per food vacuole, it follows that both deficiencies contribute to the ingestion of less total bacteria during the interfission interval. Estimates of the total number of bacteria ingested per interfission interval show that, in general, aged cells ingest one-half to one-third the total number of bacteria per cell cycle than young cells (Table VIII). Since bacteria are the source of DNA precursors (3), these results indicate that old cells have a reduced supply of exogenously supplied DNA.

DISCUSSION

The present study provides basic information on both food vacuole formation and impaired organelle synthesis and function in aging cells. The rate of formation of food vacuoles found in the present study for young cells is consistent with values reported by others, viz., 0.8 to 1.6 food vacuoles formed per minute (3, 29). Our results, however, provide the first evidence for a change in rate of formation of food vacuoles during the interfission cycle. It had previously been assumed that since

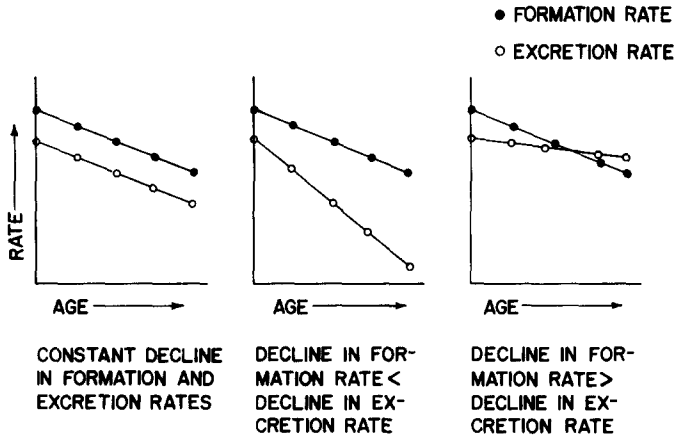

FIGURE 6 Models varying the decline in formation and excretion rates as cellular age increases. The graphs illustrate the consequences of varying the relative rates of decline in the formation and excretion rates of food vacuoles as a function of cellular age. When both rates decline at a constant rate, the difference between the rates remains constant. When the decline in formation rate is less than the decline in the excretion rate, the difference between the two rates becomes larger. Only when the decline in the formation rate is greater than the decline in the excretion rate, does the difference between the two rates diminish, approach equivalence, and the rate of excretion ultimately exceed the formation rate. Only the latter graph is consistent with our data.

TABLE V

Starvation of Cells in the Presence of Excess Food

	Percentage of cells with food vacuoles*				
	No. of food vacuoles				
Age (fissions)	None	One	Two	Three	More than three
30	0	0	0	0	100
60	1	0	0	0	99
90	0	0	1	1	98
120	2	0	0	0	98
150	1	2	1	3	93
160	12	7	6	8	67

* A random sample of 200 cells of a given fission age was examined for the number of food vacuoles present. Cells with no food vacuoles were considered starving. Those with one, two, or three food vacuoles would be likely to yield daughters with no food vacuoles.

the number of labeled food vacuoles increases at a constant rate during the interfission period, the rate of formation of food vacuoles is constant (3). The apparent constant increase in numbers of labeled food vacuoles during the interfission cycle when cells are maintained in labeled bacteria is in fact the result of an increasing rate of food vacuole synthesis. The increase in the rate of food vacuole formation is not observed since initially both labeled and unlabeled vacuoles are excreted, whereas, later in the cell cycle, only labeled vacuoles are present to be excreted. The net effect is that fewer labeled vacuoles are excreted in the

TABLE VI

Volume of Food Vacuoles

	Age (fissions)			
	40	80	120	160
Volume* (μm^3)	380	290	256	260

* The sample size was 60 cells per age in three pooled experiments. The average food vacuole volume of aged cells was not significantly different from that of young cells using the Mann Whitney U statistic.

TABLE VII

Number of Polystyrene Beads per Food Vacuole as Age Increases

	Age (fissions)				
	7	51	85	109	162
No. beads per food vacuole*	480	383	284	226	143

* Each value represents the mean number of beads per food vacuole in a minimum of 48 food vacuoles for each age examined. The student's *t*-test was used to compare the differences observed. Those averages not underlined by the same line are very significantly different from each other ($P < 0.05$).

TABLE VIII

Estimate of the Number of Bacteria Ingested per Interfission Interval

Experiment no.	Age (fissions)	No. bacteria per ml*	Food vacuole formed per min‡	Interfission time (min)§	Total no. vacuoles formed‖	No. bacteria per FV¶	Total no. bacteria ingested**
1	44		0.86	330	284	984	2.8×10^5
	148		0.50	450	225	478	1.1×10^5
2	39		0.77	370	285	398	1.1×10^5
	169	2×10^8	0.50	485	243	207	5.0×10^4
3*a*	32		0.77	330	254	574	1.5×10^5
	160	2×10^8	0.46	450	207	218	4.5×10^4
3*b*	32		0.77	330	254	916	2.3×10^5
	160	4×10^8	0.46	450	207	323	6.7×10^4

* In all experiments, the two clones compared were placed in the same suspension of bacterized Cerophyl medium. In the first experiment, the concentration of bacteria was not determined. Bacterial concentration in subsequent experiments was determined by bacterial plating. Since bacterial ingestion was an important variable, comparisons between all experiments cannot be made.

‡ The rate of formation of food vacuoles was determined by counting the number of colored food vacuoles formed in 10 min from mass cultures of cells. This value divided by 10 equals the number of food vacuoles per minute.

§ The interfission interval was the time interval from one cell division to the next.

‖ The total number of food vacuoles is the value when food vacuoles formed per minute is multiplied by the interfission time (min).

¶ The total number of bacteria per food vacuole (FV) was counted directly in a minimum of 18 food vacuoles per clone. Within each experiment, the differences in the number of bacteria per food vacuole were very significantly different ($P < 0.01$) using the Mann Whitney U statistic (37).

** The total number of bacteria ingested represents the number of bacteria per food vacuole × the total number of food vacuoles.

earlier interval than the later, thus masking a change occurring in the rate of food vacuole synthesis.

Endocytic activity, when monitored for both the ability to form and excrete food vacuoles and the capacity to ingest bacteria, was found to decrease as a function of increasing clonal age. The data offer the first evidence for a means to change the intracellular environment under constant external environmental conditions; during their interfission intervals, aging cells ingest fewer bacteria than young cells when exposed to the same external environmental conditions.

A reduced rate of formation of food vacuoles may be a result of: (*a*) reduced *de novo* synthesis of food vacuole membrane and/or (*b*) reduced recycled membrane. Regarding the latter possibility, there is extensive evidence that a major source of membrane used to form new food vacuoles is recycled food vacuole membrane (1, 2, 9, 20, 23, 24). Hence, once fewer food vacuoles are formed, fewer food vacuoles can be excreted and therefore recycled, tending to perpetuate the reduced numbers of food vacuoles. Preformed cell structure has already been considered important in cell heredity (42), and in the current study, its role in cell viability is emphasized. Regardless, the formation and excretion rates must be precisely regulated in favor of the formation rate and, as demonstrated, the advantage of the formation rate over the excretion rate is gradually lost with increased clonal age. The regulation of rates of synthesis and degradation of macromolecules as well as organelles may be considered a model of a cellular mechanism to time the occurrence of life cycle changes. When the opposing rates become equal, the components will become diluted out, thus changing the intracellular environment and then gene expression. Unlike dilution models which require exorbitant quantities of essential molecules to be present in the fertilized cells to effect phenotypic changes many cell divisions later (13, 27), this model is relatively independent of initial concentrations of molecules or organelles and dependent rather on relative synthesis and degradation rates of the critical components.

Our observation that aged cells ingest fewer bacteria per food vacuole than young cells may result from: (*a*) gullet damage; i.e., irreparable gullet damage has been identified as a source of intraclonal variation (10, 11, 34); (*b*) reduced or inefficient ability to sweep bacteria into the gullet; or (*c*) loss of membrane recognition. The latter is consistent with the idea that cell membrane alterations may occur in aging cells (45).

The observed reduction in incorporation of labeled precursors into nucleic acids both in aging paramecia (19, 36) and in aging human cells (28) could be a reflection of reduced endocytic capacity (either phagocytic or pinocytic activity). Likewise, loss of macronuclear DNA with increased clonal age (19, 31, 32) could be a result of reduced endocytic function or vice versa. However, reduction in both DNA and endocytic activity may be results of another variable (e.g., the occurrence of cell division before a sufficient increase in DNA and food vacuole numbers).

It is well known that aged cells undergo autogamy in the presence of excess food, violating the usual rule that cells must be starved to undergo autogamy (38, 40). The detection of aged cells with no food vacuoles in the presence of excess food would explain the ability of aged cells to undergo the process; the aged cells are starving despite the presence of excess food. However, starvation alone is not a sufficient cause for autogamy; cells must also be competent (40). Thus, some aging cells may be incapable of undergoing the process to rejuvenate themselves and suffer the consequences of starvation.

Starvation for nucleic acid precursors can greatly increase mutation rates (4). Since paramecia cannot form their own nucleic acids from simple precursors (5), nucleotides for macronuclear DNA are supplied by bacteria in those cultures utilizing bacteria as the food source (3). There is also evidence that starvation and aging can be correlated with increased lysosomal activity in other organisms (6, 7), in paramecia undergoing autogamy (30), and in sensitive paramecia responding to killer particles (18). Since DNase is prominent among the lysosomal enzymes, it is possible that reduced endocytic capacity may lead to released lysosomes and contribute to genetic damage as has been postulated for other aging systems (16). Aged paramecia do show micronuclear mutations after 80 fissions (43), and death at autogamy and nonvigorous clones at earlier fissions have been reported (8), but in the latter studies conjugation experiments have not yet been done to prove that the results were due to nuclear mutations. That induction of mutations may result from organelle damage or reduced endocytic function is purely speculative, but the observation that some cells in the population are starving makes this plausible.

Our study shows that aged cells lose endocytic capacity and therefore have a changed intracellular environment under constant external environmental conditions. If our data are applicable to multicellular organisms, it would be expected that aged organisms would show reduced endocytic capacity. Indeed, a significant reduction in endocytic ability as a function of age has been found in rats (44) and in man (46). Since the process of vacuolization and digestion of cellular wastes (autophagy) is a form of removal of wastes in both dividing and nondividing cells of higher organisms, reduction in endocytic or exocytic capacity could contribute to the inability of cells to remove toxic products, thereby affecting survival of both mitotic and postmitotic aging cells. Loss of autophagic ability in aging cells has already been suggested as a contributor to cellular aging (7).

The present data emphasize that nonnuclear damage could occur and be self-perpetuating and suggest that organelle damage could lead to nuclear damage. As Sonneborn (41) pointed out, "The concept of the genetic systems needs to be broad enough or, if you will, complex enough to include indirect as well as direct self-reproduction and mutability. If the original genetic material was the gene and it later created the cytoplasm, then not only has the created become able -Pygmalion-like- to control its creator, but together the two have evolved, at least in cellular heredity, into a genetic complex on a higher level of integration." He also stated that "the ultimate task will be to reunite the nuclear and cytoplasmic components of the genetic system in an integrated interreactional conception of the genetic system of the cell and to study the function of each in the work of the whole" (39). It is our view that this complex aspect of cell genetics must be involved in cell differentiation and aging, and the role of the cytoplasm in cellular aging has been emphasized in the present investigation.

The authors wish to express their sincere appreciation to Martha Jo Wagner Paris, for editing the manuscript, Meredith Russell and Michael Snyder for their excellent technical assistance, and Dr. Lyman McDonald of our

department for his statistical consultation. Gratitude is also expressed to Dr. David Nanney, University of Illinois and Dr. John Preer, Indiana University for suggestions in preparation of this manuscript.

This work was supported in part from National Science Foundation grant BMS 7501165 and the Arts and Science Division of Basic Research, University of Wyoming.

This paper is dedicated to T. M. Sonneborn on the occasion of his seventieth birthday.

Received for publication 12 March 1976, and in revised form 8 June 1976.

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