

QUANTITATIVE MEASURES OF AGING IN THE NEMATODE *CAENORHABDITIS ELEGANS*.

I. POPULATION AND LONGITUDINAL STUDIES OF TWO BEHAVIORAL PARAMETERS

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SUMMARY

As a first step in the quantitative characterization of senescence in the nematode *Caenorhabditis elegans*, we have studied movement wave frequency, defecation frequency, and whole-body water efflux as a function of age. Populations of *C. elegans*, strain N2, were cultured monoxenically on *E. coli* lawns at 20 °C. The median lifespan in such populations was approximately 12 days. Population mean movement wave frequency declined linearly with age (slope = -4.66 waves/minute per day). The decline in population mean defecation frequency (defecations per minute) was multiphasic, consisting of (1) a rapid decline (slope = -0.233 defecations/minute per day) from day 3 to day 6, (2) no apparent trend from day 6 to day 9, and (3) a gradual decline (slope = -0.089 defecations/minute per day) from day 9 to day 14. Animals alive on or after day 15 were not observed to defecate. In longitudinal studies, individual animals exhibited linear declines in movement wave frequency and multiphasic declines in defecation frequency. For future population studies, the age-dependent declines in movement and defecation frequency appear sufficiently large and reproducible to contribute to a multiparametric description of senescence in *C. elegans*.

One physiological parameter, ${}^3\text{H}_2\text{O}$ efflux, was found to be age-independent and to consist of two first-order rates. The half-times of the slow and fast efflux rates were ~15 minutes and ~2.1 minutes, respectively. The two half-times and the fractions of ${}^3\text{H}_2\text{O}$ exhibiting the two half-times were invariant with age.

INTRODUCTION

Several species of nematodes, currently used in aging research [1–10], offer many advantages as model organisms. The common advantages include a short lifespan, simple and reproducible culture conditions, and distinct signs of senescence [11]. In addition, one of these species, *Caenorhabditis elegans*, offers further, genetic, advantages due to its mode of reproduction. Because *C. elegans* is a self-fertilizing hermaphrodite, individuals

isolated from nature should be homozygous at virtually all loci, obviating the need to inbreed, and large populations of isogenic organisms can be easily obtained. In consequence, it is possible to conduct aging studies on large populations which have a uniform genetic background. Also, the property of self-fertilizing hermaphroditism has been exploited to generate a large number of mutations [12, 13], some of which can be useful in the study of aging [10].

The further exploitation of these advantages for the study of aging is, in our view, currently limited by the relative paucity of reliable quantitative markers of senescence. For the kinds of studies we anticipate, such markers are necessary adjuncts to lifespan as measures of senescence. Among the potentially quantifiable age-dependent changes which have been previously described for *C. elegans*, three are the focus of this work. Croll and coworkers [5, 6] have reported an apparent age-dependent decline in forward movement (the frequency of backwardly propagated somatic waves) and the frequency of defecations, while Searcy *et al.* [7] have reported an increase in the rate of ${}^3\text{H}_2\text{O}$ efflux with age in *C. briggsae*, a close relative of *C. elegans* [14]. We show below that the first two of these age-dependent changes, movement wave frequency and defecation frequency, are quantitative markers of senescence, and that they, along with population survival data, can be included in a multiparametric index of senescence in *C. elegans*.

MATERIALS AND METHODS

Population maintenance and age synchronization

Caenorhabditis elegans, strain N2, was grown monoxenically at 20 °C on a lawn of *E. coli*, strain OP50, spread over NGM agar [12]. *E. coli* OP50, a uracil auxotroph, was used so that only a thin layer of bacteria would grow on the limiting amount of uracil contained in the NGM agar. Hermaphrodites were used in all experiments.

Age synchrony was achieved by placing 4–7 egg-laying adult animals on a fresh NGM plate and allowing them to lay eggs for 2–4 hours. Following this egg-laying period, all adults were killed and removed with a hot needle. Day 0 (zero days of age) was defined from the midpoint of the egg-laying period. The eggs were incubated at 20 °C until the resultant animals reached an appropriate developmental stage.

A control survival curve was obtained using two 50-animal cohorts generated in independent synchronizations. Animals were transferred on a sterile needle from the original egg-laying plates to fresh plates on day 3. Subsequently, all surviving animals of the original cohorts were needle transferred to fresh plates every other day to maintain an adequate food supply and age synchrony. Both cohorts were examined daily; counts of living animals were recorded and bodies of dead animals were removed to avoid confusion. Accidental deaths occurring during transfers ($\leq 5\%$ for any population) were included in the data. An animal was classified as dead when all of the following criteria were met: (1) lack of self-initiated movement; (2) lack of pharyngeal pumping and defecation; (3) lack of any response to prodding with a needle; and (4) degradation of normal gross morphology. Transfers and counts were continued until the last animal died.

Sterilization procedure

Solutions of uridine (5 mg/ml), 5-fluorouracil (40 mM), and 5-fluorodeoxyuridine (40 mM) were filter sterilized and stored at 4 °C. Final concentrations of these compounds in NGM agar were as follows: 340 µM uridine, 400 µM 5-fluorouracil (FU), and 400 µM 5-fluorodeoxyuridine (FUDR). *E. coli* OP50 will not grow on agar containing FU or FUDR. Therefore, bacteria previously grown to stationary phase in tryptone broth were concentrated 50-fold, and 0.25 ml of this suspension was spread over the surface of these agar plates.

Animals to be treated with FU or FUDR were transferred on day 2.5 to agar plates containing the appropriate compound as described by Mitchell *et al.* [15]. Subsequent to treatment, live animals were observed at magnifications of 200X and 500X with a Zeiss compound microscope using Nomarski optics. Photomicrographs were taken on Kodak Panatomic-X film with a Nikon 35 mm camera. Movement wave frequencies of treated animals were assayed as described below.

Longitudinal study of movement rate

Wells of 6 X 4 Linbro trays were filled with 1.5 ml of NGM agar and seeded with OP50 3 days prior to use. One animal of a 3-day-old synchronous population was needle transferred to each well of a tray. Animals were maintained individually throughout the experiment by needle transfers to new wells every other day. The movement wave frequency (number of waves initiated per minute) of an animal was assayed frequently from day 3 to death using a modification of Croll's assay [16]. Assays were performed at room temperature on a 1-mm layer of movement assay agar [1.5% agar, 1.0 mM HEPES (pH 7.2), 0.25% Tween 20]. The agar was poured within 2 hours of the assays and stored covered at 4 °C until 5 minutes before use. These steps are necessary to alleviate irreproducibility in this assay due to fluctuations in the hydration of movement assay agar. While viewing through a Wild dissecting microscope, the animal was stimulated by prodding the tail with a needle and the number of waves initiated in 30 seconds was counted. This frequency was then doubled to obtain the number of waves initiated in one minute. Three assays were performed before the animal was returned to the appropriate well. Two small beakers filled with ice were placed on the microscope stage to reduce warming of the agar.

Longitudinal study of defecation frequency

Animals were maintained as in the movement wave frequency study. Defecation frequencies of all living animals were measured daily from day 3 to day 17. Measurements were made at 20 °C by observing animals in their wells and recording the time intervals between six successive defecations. Frequencies were determined only if animals were stationary and continuous pharyngeal bulb pulsation was observed throughout the entire assay period. The duration of a defecation (approximately 10 seconds) was small compared to the interval between defecations (45 seconds to 5 minutes). The average time interval was used to calculate the defecation frequency. Animals not observed to defecate in 20 minutes were assigned a defecation frequency of 0.05 defecations per minute.

$^3\text{H}_2\text{O}$ efflux assay

Nine to twelve animals of an age-synchronous population were needle transferred to a 10- μl drop of $^3\text{H}_2\text{O}$ (1 mCi/ml) suspended on the inner wall of the assay apparatus. The apparatus consisted of a cylindrical stainless steel chamber bounded at one end by a 6.5-mm diameter Mitex LC (10 μm pore) Millipore filter. The other end was attached to a 1-ml repeating syringe via a standard luer lock fitting. The volume between filter and syringe was approximately 0.4 ml. The $^3\text{H}_2\text{O}$ was buffered with sodium citrate (20 mM citrate, pH 5.0). After a 45-minute incubation, animals were washed free of excess $^3\text{H}_2\text{O}$ with 24 preflushes of 0.5 ml of BU [49.4 mM Na_2HPO_4 , 22 mM KH_2PO_4 , 68 mM NaCl (pH 6.0)]. Timing began at the first preflush with preflush 24 occurring at $t = 2$ minutes. Subsequent time samples were taken every 2 minutes for 30 minutes and every 10 minutes thereafter. Time samples consisted of 5 rapid flushes with 0.5 ml of BU. Each preflush and time sample flush was collected in a separate glass mini-vial. Following the 62-minute time sample, the apparatus was disassembled and washed, the washes being examined for animals. Preflushes, time sample flushes and washes were mixed with 4.0 ml of Aquasol-2 and counted in a Packard Tricarb scintillation counter. Counting efficiency (approximately 20%) was determined independently for each assay by counting five freshly prepared standards (1 μCi $^3\text{H}_2\text{O}$ per 0.5 ml of BU). Except for the counting, the entire procedure was performed at room temperature. Control assays were efflux assays without animals.

Optical determination of worm volume

Approximately 100 age-synchronous animals were eluted from an NGM plate and washed twice with 5 ml of BU. Animals were then treated with 1% 1-phenoxy-2-propanol in sodium cacodylate buffer (0.1 M cacodylate, pH 7.2) for 10 minutes. The diameters (at the vulva) and total lengths of 15 animals were measured at magnifications of 50X and 25X, respectively, using a Wild dissecting microscope fitted with a graticle eyepiece. By approximating animals as a cylinder ($h = 0.75$ [total length]) capped at each end by a cone ($h = 0.125$ [total length]), volumes were calculated using the average animal diameter and length and standard trigonometric equations. The standard deviation of the calculated animal volume was calculated using an appropriately derived error propagation function.

Chemicals and radiochemicals

The 5-fluorouracil and 5-fluorodeoxyuridine were obtained from Sigma Chemical Co., St. Louis, Mo. Aquasol-2 and $^3\text{H}_2\text{O}$ (1 mCi/ml) were obtained from New England Nuclear, Boston, Mass.

EXPERIMENTAL RESULTS

Age synchrony

Generating age-synchronous populations with *C. elegans* is a relatively simple process. However, *C. elegans* produces a new generation of animals every three days

(200–300 progeny per hermaphrodite), making maintenance of age synchrony difficult. Two procedures which have been used to maintain age synchrony in populations of nematodes involve separating adults from their progeny by filtration [17] or inhibiting DNA synthesis [18].

Mitchell *et al.* [15] reported that the DNA synthesis inhibitor FUDR induces sterility in *C. elegans* when administered at the onset of the reproductive period (approximately day 2.5 at 20 °C). They also reported that, except for being sterile, the treated animals appeared to be normal. Therefore, we attempted to maintain age synchrony with the FU or FUDR treatment described in Materials and Methods. Like Mitchell *et al.*, we observed that FU or FUDR treatment resulted in the cessation of production of viable eggs, while median and maximum lifespans were unaltered (see Table I). Nonetheless, photomicrographs revealed several abnormalities in FUDR-treated animals.

TABLE I
LIFESPAN DATA FOR EXPERIMENTAL POPULATIONS OF *C. ELEGANS*

| | <i>Control</i> | <i>Movement</i> ^a | <i>Defecation</i> | $^{3}\text{H}_2\text{O}$ efflux | FUDR-treated |
|-------------------------------|----------------|------------------------------|-------------------|---------------------------------|--------------|
| Median lifespan ^b | 11.4 | 11.6 | 12.2 | 11.5 | 11.5 |
| Maximum lifespan ^c | 18.6 | 18.5 | 17.0 | 20.0 | 19.0 |

^aAverages of values for movement populations A, B, and C.

^bMedian lifespan = population age (days) when 50 per cent of the cohort has died (see Fig. 2).

^cMaximum lifespan = age of last survivor at death (days).

Throughout their lifespans treated animals were shorter and narrower than untreated controls. In treated animals ovaries were granular, spermathecae were deteriorated, oocytes and eggs were disorganized, both pharyngeal bulbs were less well defined, and some vacuole-like objects were observed near the pharynx. In addition, FUDR treatment resulted in a three-fold reduction of movement wave frequency at all ages as shown in Fig. 1. Because animals treated with FUDR are demonstrably abnormal, we chose to maintain age synchrony by needle transfers of the original cohort away from its progeny (see Materials and Methods).

Lifespan

Survival data for control populations of *C. elegans*, which were manipulated only to maintain age synchrony, were obtained as described in Materials and Methods. The data for two independent populations, each containing 50 animals, were combined to give the control survival curve shown in Fig. 2. This curve yields a median lifespan of 11.5 days and a maximum lifespan of 18.6 days (see Table I). To provide a basis for comparing populations used in subsequent experiments with the composite control population, lifespan studies were performed in conjunction with each experiment (see below). As shown in Fig. 2 and Table I, the survival data for these experimental populations provide no evidence for any differences among experimental and control populations. Thus, all populations are assumed to be equivalent.

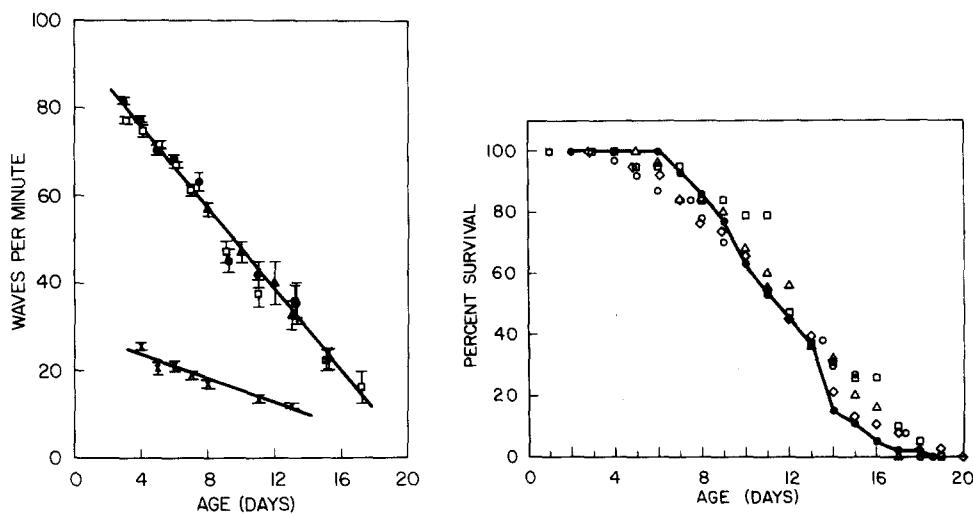


Fig. 1. Population mean movement wave frequency as a function of age. All points represent the mean \pm S.E.M. (\blacktriangle), Population A (only day 8, 10, 12 and 13 data shown for clarity); (\bullet), population B; (\square), population C; (\times), FUDR-treated population (see Materials and Methods). Lines represent linear regressions of the FUDR-treated and untreated population means.

Fig. 2. Survival curves for several populations of *C. elegans*, N2, at 20 °C. (\bullet — \bullet), Control population, $n = 100$; (\circ), movement study population A, $n = 23$; (\square), movement study populations B and C, $n = 40$; (Δ), defecation study population, $n = 25$; (\diamond), $^{3}\text{H}_2\text{O}$ efflux study population, $n \approx 38$.

The median and maximum lifespans of our populations differ from values of median (~ 16 days) and maximum (20–22 days) lifespans previously reported for *C. elegans* cultured under comparable conditions [10, 15]. However, growth and survival of *C. elegans* are dependent on temperature and quantity of food available [10, 19]. Thus, small differences in culture conditions could account for the discrepancies in the survival data noted above.

Movement wave frequency

In *C. elegans*, movement is achieved by initiating and propagating sinusoidal waves along the long axis of the body, and Croll [16] observed that these waves have a regular frequency. As previously mentioned, this frequency has been reported to be age-dependent [5, 6]; therefore, the relationship of movement wave frequency to age was quantitated.

Movement wave frequencies were measured longitudinally on separately identifiable animals as described in Materials and Methods. The three successively measured frequencies of an animal of a given age differed by 3–10 per cent. The only exceptions were obtained when one or more eggs were laid during an assay, and these assays were not included in the data. The consistency of an animal's movement wave frequency confirms Croll's observation noted above and demonstrates the reproducibility of the assay.

The results of three independent longitudinal movement studies are shown in Fig. 1; in all cases, movement wave frequency declines regularly with age. For each population, data were fitted to a straight line by the method of least squares. The individually fitted lines were virtually identical to that fitted to the data of the three populations combined ($r = -0.99$). Therefore, for populations of *C. elegans*, movement wave frequency is a linear age-dependent parameter.

To determine if movement wave frequencies of individual animals also decline linearly with age, plots of frequency *versus* age were constructed for each animal, and linear regressions were calculated for animals which were assayed on at least 4 days. The average of all correlation coefficients is -0.87 , demonstrating that movement wave frequency is linearly age-dependent for individual animals.

Additional analyses were performed to assess the possible predictive capacity of an individual's movement wave frequency. For each animal, we plotted the rate of decline in movement wave frequency, age at projected zero movement, and back-extrapolated movement wave frequency at day 0 as a function of that animal's lifespan (see Fig. 3). Figure 3 reveals that, for individual animals, there is no correlation between any of these variables and lifespan. Thus, the movement wave frequency of an individual is no better a predictor of its lifespan than is its chronological age.

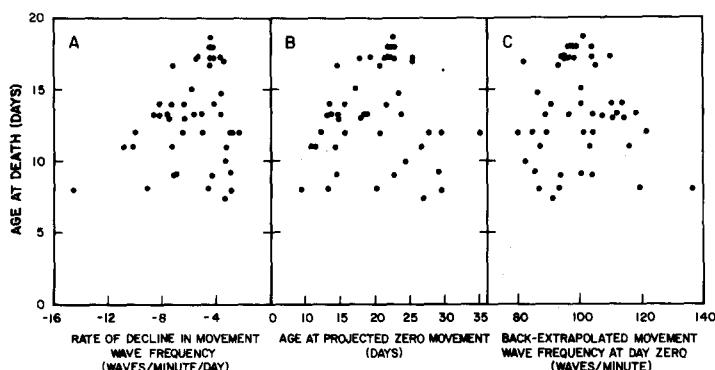


Fig. 3. Relationship between individual animal declines in movement wave frequency and lifespan. (A) Rate of decline in movement wave frequency (waves/minute per day); (B) age at projected zero movement (days); (C) back-extrapolated movement wave frequency at day zero (waves/minute). Data are for individual animals of movement populations A, B and C calculated as described in the text.

Defecation frequency

Previous reports by Croll [16] and Cassada and Russell (unpublished results) indicated that defecations occur at regular intervals. Also, Croll *et al.* [6] reported that old animals have a longer interval between defecations than do young animals. Therefore, experiments were performed to elucidate the relationship between defecation frequency and age. Defecation frequencies, based on five successive interdefecation intervals, were measured as described in Materials and Methods. On a given day, all intervals of one animal were within 5 per cent of the average interval of that animal.

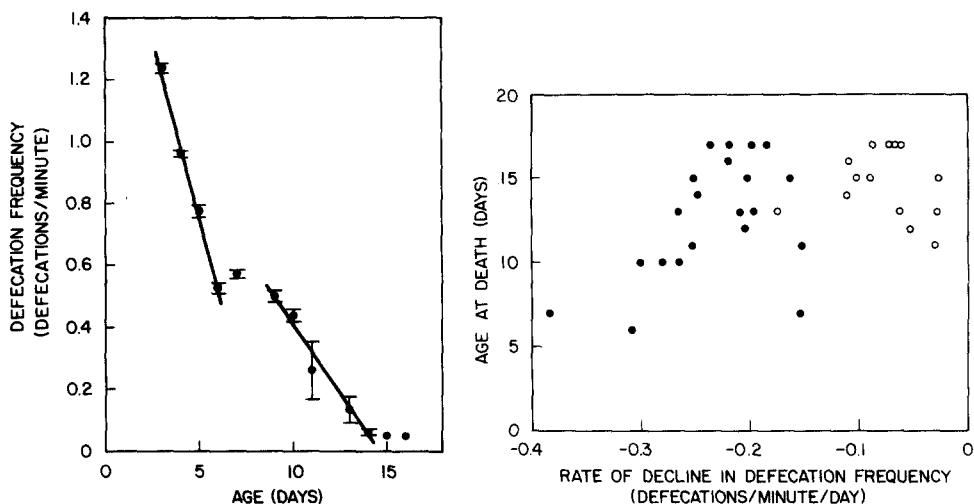


Fig. 4. Population mean defecation frequency as a function of age. All points represent the mean \pm S.E.M. Lines represent linear regressions of the day 3 to day 6 and day 9 to day 14 data, respectively.

Fig. 5. Relationship between individual animal decline in defecation frequency and lifespan. (●), Rate of decline in defecation frequency (defecations/minute per day) from day 3 to day 6; (○), rate of decline in defecation frequency (defecations/minute per day) from day 9 to day 14. Data are for individual animals of the defecation population calculated as described in the text.

As shown in Fig. 4, the decline in population mean defecation frequency with age is multiphasic. Two different rates of decline in defecation frequency are separated by a period during which there is no apparent trend. Young animals (day 3 to day 6) have a rate of decline in defecation frequency 2.5 times faster than old animals (day 9 to day 14). The regression lines calculated for young and old animals have slopes of -0.233 and -0.089 defecations/minute per day, respectively. The correlation coefficients of these two lines are -0.997 (young animals) and -0.984 (old animals), indicating a linear decline in defecation frequency with age for animals in these two age brackets.

To determine if the decline in defecation frequency was age-related for individual animals, plots of defecation frequency *versus* age were constructed for each animal. Individuals displayed the multiphasic decline in defecation frequency shown by the population. Thus, defecation frequency is an age-related parameter for individual animals.

To analyze further the relationship between defecation frequency and lifespan, regression lines of the day 3 to day 6 and day 9 to day 14 data for individual animals were calculated, and slopes (rates of decline in defecation frequency) of both lines were plotted against lifespan (Fig. 5). There is no correlation between either slope and lifespan. Similarly, no correlation was found between the *x*-intercepts (ages at projected zero defecation frequency) and lifespans or between the early and late slopes of individuals. Thus, for individual animals there is no obvious relationship between the decline in defecation frequency and lifespan, which means that the defecation frequency of an individual is no better a predictor of its lifespan than is its chronological age.

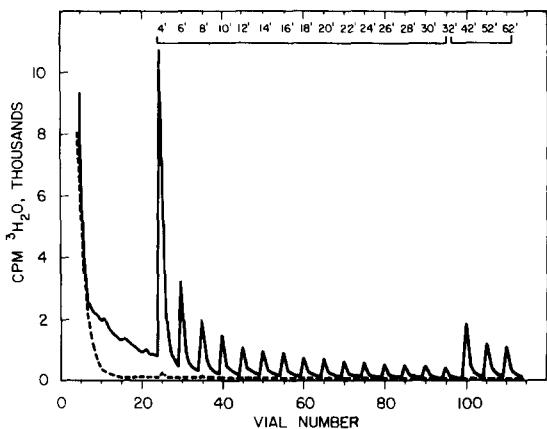


Fig. 6. Pattern of ${}^3\text{H}_2\text{O}$ recovery of a typical ${}^3\text{H}_2\text{O}$ efflux experiment (day 6 data). Vials 1 through 24 show removal of excess ${}^3\text{H}_2\text{O}$ from the apparatus. Following vial 24 (at $t = 2$ minutes), successive groups of five rapid flushes were initiated at the times indicated above the peaks. Thus, about 1.75 minutes elapsed between vials 39 and 40 and about 9.75 minutes elapsed between vials 99 and 100. (—), Experiment with 10 animals; (---), no animals present.

${}^3\text{H}_2\text{O}$ efflux

Following the lead of Searcy *et al.* [7], ${}^3\text{H}_2\text{O}$ efflux was the next parameter studied. ${}^3\text{H}_2\text{O}$ efflux was measured using the assay described in Materials and Methods. Separate subpopulations were assayed on day 3, 6, 9, 12 and 15, and Fig. 6 shows the pattern of ${}^3\text{H}_2\text{O}$ efflux typical of the assays either with (experimental) or without (control) animals present.

Radioactivity in experimental samples was corrected for control assay and background counts and transformed into counts per minute of ${}^3\text{H}_2\text{O}$ remaining in animals as a function of time (see Fig. 7). When plotted semilogarithmically, the data indicate more than one ${}^3\text{H}_2\text{O}$ efflux rate. The data from 12–32 minutes appear colinear for all assays (average correlation coefficient of –0.998) and are assumed to represent the slowest rate. To determine the number and characteristics of faster efflux rates, the calculated contributions of the slow rate were subtracted from the 2–10 minute data (see legend to Fig. 8). A semilogarithmic plot of these differences *versus* time (Fig. 8) is consistent with the interpretation that most of this remaining radioactivity exhibits a single fast efflux rate.

The half-times of the fast and slow rates were calculated from the slopes of the respective regression lines, and the fraction of total ${}^3\text{H}_2\text{O}$ exhibiting the slow efflux rate was obtained directly from the *y*-intercept of the 12–32-minute data regression line (see Fig. 7). Table II lists the results of such calculations for all assays. These data indicate that the ${}^3\text{H}_2\text{O}$ efflux half-times and relative sizes of the fast and slow “compartments” do not change between days 3 and 15. Thus, by this assay, ${}^3\text{H}_2\text{O}$ efflux is not measurably age-dependent.

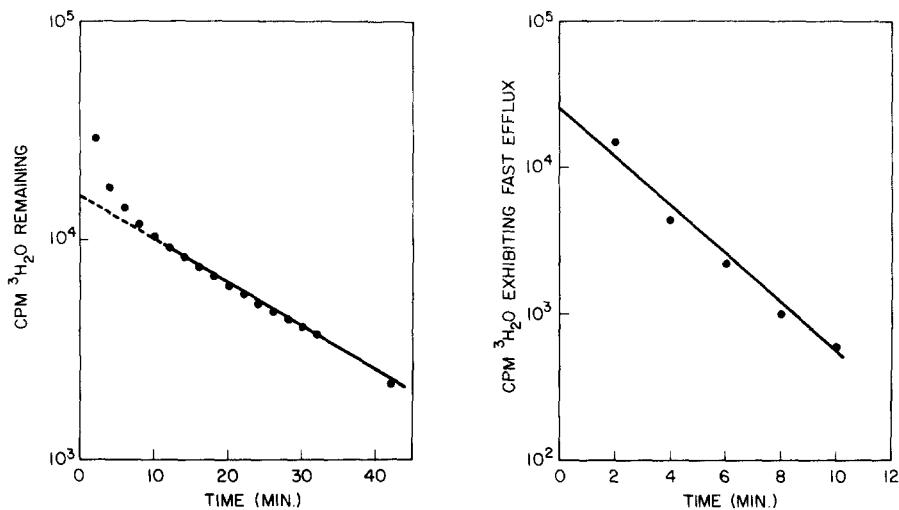


Fig. 7. Counts per minute (cpm) of ${}^3\text{H}_2\text{O}$ remaining in animals as a function of time (day 6 data). Time sample counts were corrected for background and experiment with no animals (see Fig. 6). Points represent $R_\infty - R_t$, where R_t is the sum of all time sample counts preceding time = t , and R_∞ is the sum of all time sample counts. Line represents the regression of the 12–32 minute data (see text).

Fig. 8. Determination of the fast ${}^3\text{H}_2\text{O}$ efflux half-time for the day 6 data. The 2–10 minute data were corrected by subtracting contributions from the slow efflux component (see back-extrapolation, Fig. 7). Line represents the regression of the corrected 2–10-minute data.

TABLE II
SUMMARY OF ${}^3\text{H}_2\text{O}$ EFFLUX ASSAY DATA

| Age (days) | Half-time of ${}^3\text{H}_2\text{O}$ efflux (min) | | Fraction of ${}^3\text{H}_2\text{O}$ exhibiting slow half-time ^c |
|---------------|--|-----------------------------|---|
| | Slow component ^a | Fast component ^b | |
| 3 | 14.3 | 2.1 | 0.63 |
| 6 | 15.1 | 2.1 | 0.59 |
| 9 | 15.8 | 2.1 | 0.57 |
| 12 | 14.8 | 2.4 | 0.58 |
| 15 | 16.8 | 2.1 | 0.56 |

^aCalculated from regression line exemplified in Fig. 7.

^bCalculated from corrected regression line exemplified in Fig. 8.

^cy-intercept of regression line exemplified in Fig. 7 divided by R_∞ .

Total exchangeable water volumes, calculated from ${}^3\text{H}_2\text{O}$ efflux assay data (R_∞), were compared to total body volumes calculated from measured animal dimensions (see Materials and Methods). As shown in Fig. 9, this comparison reveals no significant differences. This suggests that: (1) the 45-minute labeling period is sufficiently long to equilibrate most, if not all, of the body volume with ${}^3\text{H}_2\text{O}$; and (2) no significant amount of internalized radioactivity is lost or gained during preflushing.

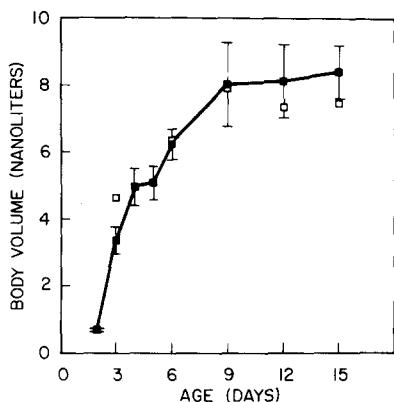


Fig. 9. Animal volume as a function of age. (□), Volumes calculated from ${}^3\text{H}_2\text{O}$ efflux data (R_∞); (●), mean volume \pm S.D. calculated from measured animal dimensions (see Materials and Methods).

Survival of assayed populations

As previously discussed, control and experimental populations have nearly identical survival curves and median and maximum lifespans (see Fig. 2 and Table I). The survival data for the populations used in the movement wave frequency and defecation frequency studies were derived from the same animals assayed for movement and defecation. Thus, the repeated needle transfers and other manipulations during movement and defecation assays do not affect the survival characteristics of populations of *C. elegans*.

The survival data for the population employed in the ${}^3\text{H}_2\text{O}$ efflux study were obtained from a subpopulation independent of those subpopulations assayed for efflux. Therefore, in order to assess directly the effects of the efflux assay on survival, the survivors of the days 3 and 6 assays were recovered and cultured at 20 °C on NGM plates. The day 3 and day 6 survivors had median lifespans of 12 and 13.5 days, and maximum lifespans of 19 and 18 days, respectively. These results indicate that the ${}^3\text{H}_2\text{O}$ efflux assay also does not affect the survival characteristics of populations of *C. elegans*.

The lack of any apparent effect of these assays on survival implies that animals are not adversely affected by the manipulations involved in performing the assays. This is supported further by the observation that the survivors of the day 3 efflux assay produced approximately normal numbers of viable progeny. Thus, it should be possible to assay populations of *C. elegans* for movement wave frequency, defecation frequency and/or ${}^3\text{H}_2\text{O}$ efflux without altering the subsequent survival of the members of the populations.

DISCUSSION

The results reported above demonstrate that two behaviors of the nematode *C. elegans* – movement and defecation – exhibit quantitative age-dependent changes which can be straightforwardly assayed in individuals and which occur with sufficient regularity

to make them useful as quantitative markers of senescence. These results are thus in good general agreement with an earlier report by Croll *et al.* [6], in which changes of this kind were first investigated in *C. elegans*.

Comparison to previous results

With respect to movement, Croll *et al.* [6] showed that in axenically cultivated populations, the maximum frequency of backward waves declined from approximately 110 waves/minute at day 4 to approximately 20 waves/minute at day 20, with a phase of approximately linear decline from day 8 to day 16 (median lifespan = 17.6 days). These measurements were conducted on animals in liquid, without apparent stimulation. Our observations on monoxenically grown animals differ in that the wave propagation rate was measured on an agar surface after a transfer, which provides maximal stimulation for a period of at least 30 seconds. The greater physical constraints experienced by an animal on an agar surface no doubt account for the fact that our maximum wave propagation rates are approximately 80 waves/minute and not 110. However, we would expect our rates to be otherwise comparable to the rates observed by Croll *et al.* [6]. Indeed, we also find a linear decline in population mean movement wave frequency with age from day 3 through day 17 (median lifespan = 11.6 days). Individual animals also exhibit regular and approximately linear declines in movement wave frequency with age. (Croll [5] has separately noted that the probability that a given unstimulated animal will move during a short observation interval also declines with age.)

Croll *et al.* [6] described age-dependent decreases in the mean defecation frequency for both axenically and monoxenically grown *C. elegans*. The defecation frequencies for both kinds of culture exhibited closely parallel changes through day 10, declining gradually from day 4 to day 8 and then quite sharply between days 8 and 10. The axenic culture subsequently showed little if any significant change in defecation frequency. Our results on monoxenically grown animals confirm the general direction of these changes but not the temporal details. We find a relatively rapid decline in defecation frequency between days 3 and 6, followed by a more gradual decline thereafter. This is a pattern of change resembling that found in the axenic culture of Croll *et al.* [6] but occurring at earlier times. Since our median lifespan for this experiment was 12.2 days, *versus* 17.6 days for Croll *et al.*'s [6] axenic culture, and since we routinely observe reproduction to cease between days 5 and 6, whereas Croll *et al.* [6], comment that oviposition ceased between days 8 and 10 in their axenic cultures, we suggest that our monoxenic conditions simply permit an accelerated overall life cycle relative to the axenic conditions used by Croll *et al.*

Our investigation of ${}^3\text{H}_2\text{O}$ efflux rates in *C. elegans* was prompted by an earlier study of ${}^3\text{H}_2\text{O}$ efflux by Searcy *et al.* [7] in the closely related species *Caenorhabditis briggsae*; our conclusions differ from those of Searcy in important ways. Some of the differences may conceivably be attributable to the difference in species studied or to the different culture conditions used (monoxenic *versus* axenic), but at least one difference is probably methodological in origin, and another is a difference in interpretation. First, Searcy *et al.* [7] report that for animals of a given age ${}^3\text{H}_2\text{O}$ efflux could be described as

a process with a single first-order rate constant. However, we find that at least two processes, with rather different first-order rate constants, are necessary to describe our data. This difference we believe to be methodological. The rapidly flushable chamber used in our study was designed to facilitate the detection of exchange processes with short time constants (≥ 1 min), and our control experiments, without animals, demonstrated that the chamber itself has no measurable reservoir with exchange properties resembling those we attribute to *C. elegans* (see Fig. 6). In contrast Searcy *et al.* used [^{14}C] sorbitol (assumed not to permeate the animals) to correct for $^3\text{H}_2\text{O}$ retained in their apparatus but not inside the animals; and they state that this correction amounts to about 50%, which necessarily compromises the accuracy of the data. In our case, the comparable correction, obtained from our no-animal control, was unmeasurably low, attesting to the more thorough flushing possible in our apparatus; and congruently, our data exhibit less scatter than do those of Searcy *et al.* [7]. For all of these reasons we believe that if *C. briggsae* shows a rapid exchange process comparable to that which we observe in *C. elegans* (and we would suspect that it does) that process could well have been missed by Searcy *et al.*

The occurrence of two exchange processes in *C. elegans* suggests the occurrence of two compartments with differential access to the surrounding medium. The reality of such compartments is supported by the fact that their aggregate volume, computed from the observed radioactivity in each and the specific activity of the $^3\text{H}_2\text{O}$ used, corresponds closely to the total optically measured volume of the animals at each of five points through the life cycle (Fig. 9). In addition, the relative sizes of the two compartments remain remarkably constant throughout the life cycle (Table II), as might be expected if they represented anatomical entities. Whether the compartments indeed represent such entities, and if so which ones, remains uncertain; however, if they do, each of the two must comprise a significant proportion of total animal volume (~60% for the slowly exchanging compartment, ~40% for the rapidly exchanging one). Whether these two compartments might have different exchange rates for molecules other than water is a separate question, and one that might be relevant in studies of drugs and inhibitors whose actions depend on permeation to specific internal target sites.

A second difference between our $^3\text{H}_2\text{O}$ efflux results and those of Searcy *et al.* concerns the possibility of age-dependent changes. Searcy *et al.* [7] report, for the single exchange process they detected, a half-time of 12.6 ± 1.8 minutes for "young" (7 days) animals and 5.9 ± 2.1 minutes for "old" (21 days) animals. In contrast, we detect no age-dependent changes, whether we consider the half-time of our rapidly exchanging compartment (2.1 ± 0.1 minutes), the half-time of our slowly exchanging compartment (15.4 ± 1.0 minutes) or the distribution of radioactivity between the two compartments (Table II). This difference we believe to be one of interpretation. In deriving their values for half-time of exchange, Searcy *et al.* [7] made a correction for surface-to-volume ratio changes between young and old animals based on their detection of only a single exchange process. They assumed that the animal was behaving as a single compartment and that the external cuticle was the effective barrier limiting exchange between this compartment and the medium. From data provided by Searcy *et al.* we have rederived an

actually observed half-time of 7.3 ± 1.0 minutes for their young animals and we note that this observed value is not significantly different from the value of 5.9 ± 2.1 minutes observed for old animals.

Whether or not a surface-to-volume correction is appropriate is debatable, since such a correction is based on an unverified assumption; namely, that the cuticle is rate-limiting. In our case especially, where two exchange rates are detected, it can not be true that the same cuticle is rate-limiting for both. We believe it is preferable to use uncorrected data, free of assumptions, because any changes derived by correction would reflect measured changes in surface and volume; and without corrections our data and those reported by Searcy *et al.* provide no evidence of age-related changes in ${}^3\text{H}_2\text{O}$ efflux.

Behavioral parameters as measures of aging

For each of the two parameters that we have shown to be age-dependent — movement wave frequency and defecation frequency — population measurements show highly reproducible changes. In addition, longitudinal studies show that the major features of population changes are preserved in individuals. However, the relationship between individuals and the population is not a straightforward one.

Our data indicate that the dispersions about the population means (S.D./mean) for movement and defecation frequencies increase progressively from 7–10 per cent on days 3–5 to 50–60 per cent after day 12. This increase in dispersion with age apparently arises because individual animals differ widely in their rates of change in these parameters with age. For example, individuals' rates of decline in movement wave frequency with age show a mean = -5.7 ± 2.5 waves/minute per day and a range from -2.4 to -14.6 waves/minute per day. These differences are not due to inherent variability in the assays: two-way analysis of variance shows that differences amongst individuals are highly significant ($p < 0.01$), even at early times.

In view of the virtual genetic homogeneity ensured by *C. elegans*' mode of reproduction, it seems highly unlikely that such variations can be attributed to genetic differences between individuals. If one accepts the postulate that there is an underlying aging process which is quite reproducible in individuals of uniform genetic background, then a high degree of variation in measures of senescence must be understood as resulting from loose coupling between the underlying process and its ultimate consequences. Such a view might be summarized as one in which the underlying process is linked probabilistically, and with varying degrees of tightness, to resultant senescent changes. In this view, senescent changes which are themselves only loosely linked to the underlying process should be rather poor predictors of each other in individuals. Figures 3 and 5 reveal that this appears to be the case for movement wave frequency, defecation frequency and survival.

Interestingly, lifespan, often used as a measure of senescence, is in this view only a loosely linked parameter. At 5 days of age, for example, the fractional standard deviation in lifespan is 25 per cent of the population mean, compared to about 10 per cent for movement and defecation. It follows that a better measure of a putative underlying mechanism of aging might be obtained not by using lifespan as a sole criterion, but rather

by constructing a multiparametric index to which other parameters, some of which may be more tightly linked to the underlying process, could contribute.

An example of a multiparametric index of senescence was provided by Hollingsworth *et al.* [20], using a battery of nine age-related parameters for humans. The independent relationships of these nine parameters with chronological age were weak, having correlation coefficients ranging from +0.604 to -0.423. They then calculated a composite "physiological age", using all nine parameters simultaneously, and compared this "physiological age" to chronological age. The resultant correlation coefficient for this relationship was considerably improved, being slightly less than +0.90. Moreover, if chronological age was limited to ages greater than or equal to 35 years, the correlation coefficient for the relationship between composite "physiological age" and chronological age became nearly +1.0.

In a first attempt to construct a primitive index of "physiological age" for *C. elegans*, we have explored a model in which physiological age, α , is considered to be a linear function of survival (S), movement wave frequency (M), and defecation frequency (D); that is,

$$\alpha = \alpha_0 + \alpha_1(S) + \alpha_2(M) + \alpha_3(D).$$

From the data given in this paper, using data from only those days on which all three parameters were measured, we calculated the regression coefficients by the method of least squares. The resulting equation is

$$\alpha = 18.08 - 0.05S - 0.085M - 2.47D$$

where S is expressed as percentage survival (control population, Fig. 2), M is expressed as waves/minute (population B, Fig. 1) and D is expressed as defecation frequency (Fig. 4). The relative influences of the three parameters on the calculated values of α are: survival, 0.35; movement, 0.40; defecation, 0.21. Values of α for each day were calculated using the actual data obtained on that day.

A plot of physiological age, α , against chronological age, t , is shown in Fig. 10A. It is evident that α is closely colinear with t . For comparison, Fig. 10B shows a physiological age estimator, α_M , calculated using population mean movement wave frequency alone. Although α_M is also reasonably colinear with t , as would be expected by examining Fig. 1, the three-parameter estimator, α , is clearly superior as judged by the reduced standard deviations at all ages.

As noted by Comfort [21] and Hollingsworth *et al.* [20], this method requires that the parameters used be sufficiently diverse that they may be properly considered as independent variables. The data of Fig. 3 indicate that movement and survival are independent of each other, and Fig. 5 shows that defecation and survival are also independent, but we have not yet shown that movement and defecation are uncorrelated in individuals.

Clearly, such an estimator, α , could be expanded by the addition of other parameters, with appropriate tests for independence. At a minimum, the inclusion of additional parameters would be expected to reduce the error in α . The true utility of multi-

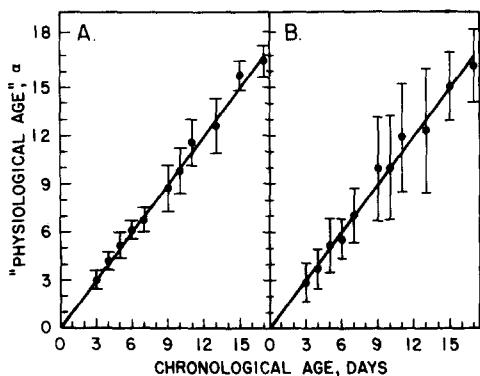


Fig. 10. Relationship between estimators of "physiological age" and chronological age. The lines represent ideal correspondence between α and t . (A) $\alpha = 18.08 - 0.05S - 0.085M - 2.47D$, calculated using population mean values for each day. Error bars show standard deviations in α calculated using the standard deviations in population means for movement and defecation, weighted by the corresponding regression coefficients. No error was assigned to population survival measurements. (B) $\alpha = 19.68 - 0.206M$, calculated from data for population B (Fig. 1).

parametric estimators will depend on their response to environmental factors such as temperature or nutritional restriction [10], which may affect the rate of a possible underlying process of aging. This "rate of aging" would be represented by $d\alpha/dt$, with α calculated using the regression coefficients determined for a standard state (for example, monoxenic plate cultures at 20 °C). If α included a sufficiently large number of independent parameters and if the posited underlying process really exists, $d\alpha/dt$ would be little affected by environmental factors which altered individual outputs from the underlying process of aging. Conversely, environmental factors which substantially altered $d\alpha/dt$ without increasing the dispersion in α would be inferred to have influenced a common underlying process.

While this mode of analysis is clearly preliminary, we believe that the results reported in this paper are sufficiently encouraging to warrant the further development and testing of such a multiparametric index of aging in *C. elegans*.

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REFERENCES

- 1 M. Erlanger and D. Gershon, Studies on aging in nematodes: II. Studies on the activities of several enzymes as a function of age. *Exp. Gerontol.*, 5 (1970) 13–19.

- 2 B. M. Zuckerman, S. Himmelhoch, B. Nelson, J. Epstein and M. Kisiel, Aging in *Caenorhabditis briggsae*. *Nematologica*, 17 (1971) 478–487.
- 3 B. M. Zuckerman, B. Nelson and M. Kisiel, Specific gravity increase of *Caenorhabditis briggsae* with age. *J. Nematol.*, 4 (1972) 261–262.
- 4 B. M. Zuckerman, S. Himmelhoch and M. Kisiel, Fine structure changes in the cuticle of adult *Caenorhabditis briggsae* with age. *Nematologica*, 19 (1973) 109–112.
- 5 N. A. Croll, Indolealkylamines in the coordination of nematode behavioral activities. *Can. J. Zool.*, 53 (1975) 894–903.
- 6 N. A. Croll, J. Smith and B. M. Zuckerman, The aging process of the nematode *Caenorhabditis elegans* in bacterial and axenic culture. *Exp. Aging Res.*, 3 (1977) 175–199.
- 7 D. G. Searcy, M. J. Kisiel and B. M. Zuckerman, Age-related increase of cuticle permeability in the nematode *Caenorhabditis briggsae*. *Exp. Aging Res.*, 2 (1976) 293–301.
- 8 R. Hosono, Age dependent changes in the behavior of *Caenorhabditis elegans* on attraction to *Escherichia coli*. *Exp. Gerontol.*, 13 (1978) 31–36.
- 9 M. Rothstein, Altered enzymes in the free living nematode *Turbatrix aceti* aged in the absence of fluorodeoxyuridine. *Mech. Ageing Dev.*, 8 (1978) 175–184.
- 10 M. R. Klass, Aging in the nematode *Caenorhabditis elegans*: Major biological and environmental factors influencing life span. *Mech. Ageing Dev.*, 6 (1977) 413–429.
- 11 D. Gershon, Studies on aging in nematodes: I. The nematode as a model organism for aging research. *Exp. Gerontol.*, 5 (1970) 7–12.
- 12 S. Brenner, The genetics of *Caenorhabditis elegans*. *Genetics*, 77 (1974) 71–94.
- 13 D. L. Riddle, The genetics of development and behavior in *Caenorhabditis elegans*. *J. Nematol.*, 10 (1978) 1–16.
- 14 P. A. Friedman, E. G. Platzer and J. E. Eby, Species differentiation in *Caenorhabditis briggsae* and *Caenorhabditis elegans*. *J. Nematol.*, 9 (1977) 197–203.
- 15 D. H. Mitchell, J. W. Stiles, J. Santelli and D. R. Sanadi, Synchronous growth and aging of *Caenorhabditis elegans* in the presence of fluorodeoxyuridine. *J. Gerontol.*, 34 (1979) 28–36.
- 16 N. A. Croll, Components and patterns in the behavior of the nematode *Caenorhabditis elegans*. *J. Zool.*, 176 (1974) 159–176.
- 17 M. J. Tilby and V. Moses, Nematode aging. Automatic maintenance of age synchrony without inhibitors. *Exp. Gerontol.*, 10 (1975) 213–223.
- 18 M. J. Kisiel, B. Nelson and B. M. Zuckerman, Effects of DNA synthesis inhibitors on *Caenorhabditis briggsae* and *Turbatrix aceti*. *Nematologica*, 18 (1972) 373–384.
- 19 L. Byerly, R. C. Cassada and R. L. Russell, The life cycle of the nematode *Caenorhabditis elegans*: I. Wild-type growth and reproduction. *Dev. Biol.*, 51 (1976) 23–33.
- 20 J. W. Hollingsworth, A. Hashizume and S. Jablon, Correlations between tests of aging in Hiroshima subjects – an attempt to define “physiological age”. *Yale J. Biol. Med.*, 38 (1965) 11–26.
- 21 A. Comfort, *The Biology of Senescence*, 3rd ed., Elsevier-North Holland, New York, 1979, pp. 299–312.