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PROTEIN DEGRADATION IN AGED NEMATODES (*TURBATRIX ACETI*)

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SUMMARY

The rates of degradation of total soluble proteins in the free-living nematode, *Turbatrix aceti*, have been estimated by following the loss of acid-insoluble radioactivity from protein during a nonradioactive chase period after initial labeling with [^{35}S]methionine. These proteins appear to lose label kinetically as a homogeneous class in age-synchronized nematode populations. However, proteins are degraded more slowly in senescent cultures than in young cultures. Protein degradation rates decline progressively during nematode aging. These findings suggest that the protein degradative system in *T. aceti* may become partially defective with advancing age which may result in the accumulation of aberrant protein molecules in senescent organisms.

Substantial evidence is now available documenting the existence of faulty enzyme molecules in senescent organisms. Catalytically incompetent forms of several enzymes have been detected in a variety of phylogenetically distant organisms, including nematodes (1-7), mice (8, 9), rats (10) and rabbits (11). Although the physicochemical abnormalities in protein structure which prevent the maximum expression of catalytic activity remain ill-defined, the accumulation of such abnormal enzyme species in cells could potentially have widespread deleterious effects on normal cellular function and thus contribute significantly to the process of biological aging (12, 13).

It has long been known that proteins in eukaryotic and prokaryotic cells are in a dynamic state, undergoing continuous replacement by the process of protein turnover. The contribution of turnover as a mechanism for regulating

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the concentration of proteins in tissues is of particular interest with regard to the age-related accumulation of defective enzyme molecules, since recent evidence indicates that this process may play an important role in the rapid elimination of error-containing protein (12, 14). If this is so, then the demonstrated presence of abnormal enzymes in aged cells may be indicative of an age-associated decrease in the efficiency of the protein degradative system.

We have attempted to assess the effect of aging on the stability of the protein degradative machinery by comparing relative rates of protein degradation in age-synchronized cultures of the free-living nematode, *Turbatrix aceti*. The suitability of the nematode system for studies on aging has been described (15, 16) and the occurrence of altered enzymes in aged *T. aceti* has been well-documented (17, 18).

MATERIALS AND METHODS

Growth and Aging of T. aceti. *T. aceti* was grown at 30° in basal medium containing 4% acetic acid (19) and aged in the presence of 5'-fluorodeoxyuridine as described by Hieb and Rothstein (16). In some experiments, aging was conducted at 36° (16) as indicated in the text.

Nematode Labeling. Organisms were harvested at various ages by filtration onto fritted glass Buchner funnels, washed with basal medium, resuspended in the same medium (2.6mM in L-methionine) at 20,000 worms/ml with the addition of L-[^{35}S]methionine (5-10μCi/ml, Amersham Searle), and incubated in the radioactive medium at 30° for 24 hr. This labeling period provided extensive isotope incorporation into protein and thus minimized errors in the measurement of acid-insoluble radioactivity.

Measurement of Protein Degradation Rates. [^{35}S]-Labeled nematodes were collected by filtration under gentle suction, washed five times with 15-20ml of basal medium containing additional unlabeled L-methionine (Sigma, 40mM final concentration), then reincubated in the methionine-supplemented medium at a concentration duplicating that of the original culture for periods up to 72 hr. The organisms were transferred to fresh medium every 8 to 12 hr during the chase period in order to minimize reincorporation of isotope. Toxic effects due to excess methionine in the chase medium were not observed.

Nematode samples were withdrawn in duplicate at appropriate time intervals, washed, and homogenized in 5ml of 0.05M potassium phosphate (pH 7.5) in an ice-cold French pressure cell (Aminco) at 18,000 p.s.i. The homogenate was centrifuged at 105,000g for one hr and Cl_3CCOOH (6% final concentration) was added to the particulate-free supernatant at 0°. The acid-precipitable residues were resuspended in 6% Cl_3CCOOH containing 0.01% L-methionine, heated at 100° for 15 min, extracted twice with absolute ethanol-ether (3:1, v/v) and twice with ether, and dissolved in 0.5N NaOH. Radioactivity in the NaOH solutions was quantified by counting aliquot samples in 10ml of scintillation fluid (125g napthalene/7.5g PPO/0.3g POPOP in 1 liter of dioxane) using a Nuclear-Chicago Mark I liquid scintillation spectrometer.

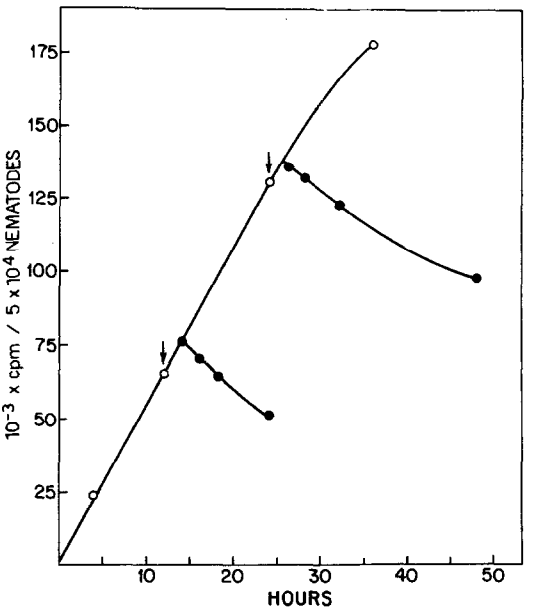

Figure 1: Incorporation of [^{35}S]methionine into soluble proteins of *T. aceti* and loss of radioactivity during chase in unlabeled medium. Nematodes (6 days of age) were incubated with [^{35}S]methionine (10 µCi/ml; 2.6 mM) at 30°. At the times designated by the arrows, portions of the culture were removed and the worms transferred, after washing, to fresh medium containing 40mM nonradioactive methionine. Protein samples were prepared at specified intervals and assayed for radioactivity as outlined in Materials and Methods. Results are expressed as the total radioactivity in acid-insoluble protein/50,000 organisms. Each point represents the mean from duplicate assays which were within ±10% of each other. (○), pulsed portions of the culture; (●), chased portions of the culture.

Counting of nematode populations was carried out by the method of Tomlinson and Rothstein (20).

RESULTS

Fig. 1 describes the incorporation of radioactivity into nematode proteins measured during a pulse-chase experiment with a 6-day old culture. After either a 12- or 24-hr labeling period, incorporation of [^{35}S]methionine is effectively arrested upon reincubation of isotopically-labeled nematodes in the presence of 40mM unlabeled precursor. Cessation of isotope incorporation occurs in 2 to 4 hr and is followed by a subsequent decline in protein-bound counts, whereas pulsed counts continue to be incorporated if unchased. This chase

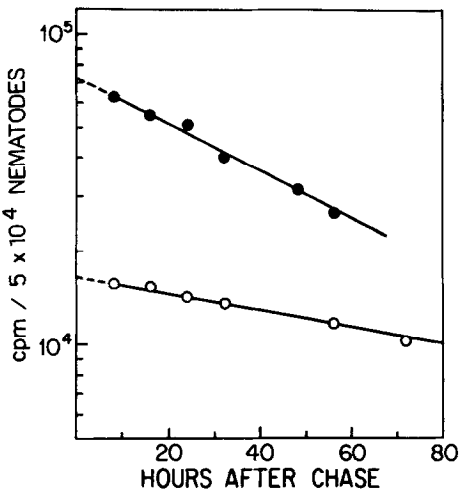

Figure 2: Chase of [^{35}S]methionine from prelabeled acid-insoluble proteins of 6-day old (●) and 16-day old (○) nematodes. *T. aceti* was incubated at 30° in the presence of [^{35}S]methionine (5 µCi/ml; 2.6mM) for 24 hr, then transferred to fresh medium containing excess unlabeled methionine as described in Figure 1. Total radioactivity in acid-precipitable protein/50,000 organisms was determined at the designated time intervals. Data points represent the means from duplicate assays which were within ±10% of each other.

procedure was found to be effective in nematode cultures at all stages of aging; measurable loss of radioactivity in proteins was observed within 8 hr after transfer to unlabeled medium.

Fig. 2 shows the time course for acid-insoluble radioactivity remaining in proteins of 6- and 16-day old nematodes during a nonradioactive chase period after prelabeling with [^{35}S]methionine for 24 hr. In both cultures, loss of label follows single exponential decay kinetics, which may indicate that proteins are degraded with similar half-lives over the time period examined. Rapidly degraded proteins with half-lives less than 8 hr (the first time point) would, of course, escape detection by this procedure. It is apparent, however, that the rate of disappearance of isotope from proteins within this restricted range is significantly diminished in the 16-day culture. The observed reduction in the extent of [^{35}S]methionine incorporation into proteins of this same

TABLE I
Rate of Degradation of Soluble Proteins as a Function of Nematode Age[a/]

Nematode Age (Days)	Rate of Degradation k_D ($hr^{-1} \times 10^3$)	Average Half-Life[b/] $t_{1/2}$ (hr)
2	28.3 ± 0.60	25
6	14.5 ± 1.45	48
11	7.68 ± 0.18	90
16	5.24 ± 0.64	132
20	2.58 ± 0.50	269

[a/] Experimental conditions are described in the legend of Figure 2. Apparent rates of protein degradation (k_D) were calculated by least squares analysis from the slopes of first-order plots for the loss of acid-precipitable radioactivity in chase experiments (Figure 2). Values of k_D are the averages of three individual culture experiments.

[b/] Calculated according to the relationship,

$$t_{1/2} = \frac{\ln 2}{k_D}$$

culture during the labeling period, as evidenced from the extrapolated intercept at the ordinate, reflects the slower rate of protein synthesis in aged nematodes (21)

Table I records data obtained from chase experiments with nematode cultures at various stages of aging. Apparent first-order rate constants for protein degradation (k_D, hr^{-1}), calculated from the slopes of semilogarithmic plots depicted in Fig. 2, decrease progressively and approximately 10-fold from 2 to 20 days of age, corresponding to an increase in mean half-life from 25 to 269 hr. A steady decline in protein degradation *in vivo* can also be demonstrated during

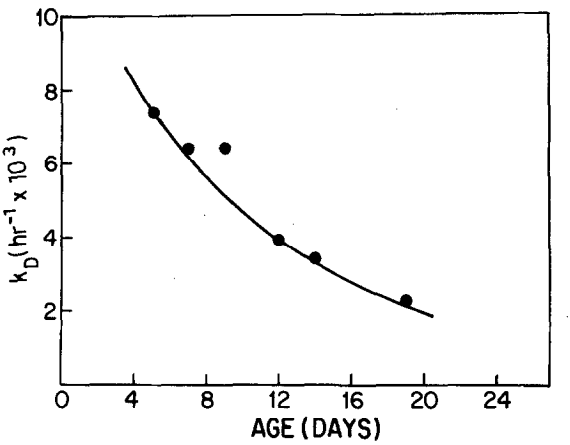

Figure 3: Variation in the apparent rate constant (k_D) for protein degradation during aging of *T. aceti* at 36° (16). Experimental conditions are outlined in the legend of Figure 2. Values of k_D are the averages of two individual culture experiments.

aging of *T. aceti* at 36° (Fig. 3). Thus, our results cannot be ascribed to the use of fluorodeoxyuridine to obtain age-synchronized nematode populations.

DISCUSSION

The present findings strongly suggest that the rate at which proteins are degraded in *T. aceti* is dramatically reduced in aged organisms. Our estimates of protein degradation rates are based upon the traditional method of measuring the loss of radioactivity from total particulate-free supernatant proteins after prelabeling with radiolabeled [^{35}S]methionine and, as such, are subject to the well-recognized complication of isotope reutilization (22, 23). Our procedure involving washing of labeled organisms and frequent changes of medium containing an excess of methionine during the chase period appears to be effective in reducing, if not completely eliminating, this complication by dilution of the radioactive amino acid pool (Fig. 1). In addition, since we are concerned with relative rates of degradation, differential isotope recycling in young and aged cultures would have to be invoked if this mechanism were solely responsible for the observed increase in protein half-lives. However, differences in amino acid pool sizes and permeability barriers, factors which could lead to different

rates of isotope reutilization, are not detectable in young and senescent animals (21). Moreover, the reduction in protein synthetic rates of aging nematodes (21) should, in fact, suppress recycling and thus mask the apparent decrease in protein degradation.

The observed loss of radioactivity from cytosol proteins (Fig. 2) presumably reflects the proteolytic breakdown of prelabeled polypeptide chains. This effect cannot be explained by the inclusion of soluble proteins in insoluble subcellular aggregates or by the excretion of intact proteins, as acid-insoluble radioactivity does not accumulate in the particulate fraction (sedimentable at 105,000g) or in the medium during the chase period. On the other hand, continual release of acid-soluble [^{35}S] counts to the medium does occur from [^{35}S]-labeled nematodes over the same time interval. However, accurate determinations of efflux rates for acid-soluble counts originating exclusively from proteolysis are precluded by a slow rate of loss of radiolabeled amino acids from internal nematode pools. This method, therefore, does not provide meaningful and reliable measurements of protein degradation rates in the nematode system.

Although the reason for the pronounced slowing of protein degradation in aging nematodes remains to be elucidated, an attractive possibility is that the protein degradative system becomes partially dysfunctional with age. A functional failure in the efficiency of this system could then lead to an accumulation of aberrant proteins in senescent animals if their degradation became rate-limiting. In this regard, it is noteworthy that the accumulation of defective nematode enzymes occurs progressively with advancing age (1, 3), as does the observed decline in protein degradative capacity (Table I and Fig. 3). Other explanations are, of course, possible. For example, senescent organisms may possess a class of abnormal proteins with longer half-lives. However, in one case examined (6), structurally altered enolase purified to apparent homogeneity from aged nematodes exhibits a greater susceptibility to proteolytic digestion *in vitro* than its "young" counterpart.

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