Journal of Cellular Biochemistry 44:153–165 (1990)

Biochemical Changes Associated With the Mechanism Controlling Superoxide Radical Formation in the Aging Rotifer

Masaaki Sawada and John C. Carlson

Lady Davis Institute for Medical Research, Sir M.B. Davis Jewish General Hospital/McGill University, 3755 Chemin Cote Ste-Catherine, Montreal, Quebec, Canada H3T 1E2 (M.S.), and Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1 (J.C.C.)

Levels of the superoxide radical (SOR) and lipid peroxides were measured and found to increase during aging in the short-lived rotifer, *Asplanchna brightwelli*. Life-span was altered by changes in environmental temperature, absence of light, diet restriction, exposure to ultraviolet radiation, and addition of vitamin E to the diet. Each of the conditions that lengthened life-span decreased SOR and lipid peroxide levels, and each condition that shortened life-span increased levels of SOR and lipid peroxides. Additional experiments indicated that on the third day of age, there was a significant increase in Ca^{2+} uptake and phospholipase A_2 activity in membrane samples and an elevation in superoxide dismutase and catalase activity in rotifer homogenates. In addition, SOR concentration was inhibited by the addition of bromophenacyl bromide and indomethacin to membrane samples. By day 5 there was also a significant increase in the lysosomal enzyme, α-mannosidase. The results of this study indicate that levels of the SOR and lipid peroxides are coupled to rotifer life-span and that activation of phospholipase A_2 may contribute to the elevation of these agents in older animals.

Key words: free radicals, lipid peroxides, life-span, calcium, phospholipase A_2, scavenging enzymes

Cellular breakdown occurs during the aging process. It is caused by a number of biochemical changes that result in a loss of function. Free radical concentration increases during aging, and it is possible that this elevation plays a central role in cellular degeneration [1]. These agents are toxic and rising levels in the cell produce detrimental changes that affect metabolic activity. For example, free radicals attack polyunsaturated fatty acids causing lipid peroxidation and disruption of organelle function [2]. Normally these agents are removed by scavenging enzymes or by antioxidants. However, in older cells increased production or decreased efficiency of detoxification may result in higher levels of free radicals, which could account for cellular degeneration and the increasing frequency of disease and the higher mortality rate in older people [1].

Received February 20, 1990; accepted July 10, 1990.

We have used the rotifer, *Asplanchna brightwelli,* an invertebrate with a 5–6 day life-span, to investigate the biochemical steps involved in cellular breakdown during aging in this model. Previous studies indicate that the life-span of this species can be altered by diet, temperature, absence of light, or UV radiation and that life-span modification is associated with changes in lipid peroxide levels [3,4]. The prospect that free radicals mediate cellular breakdown in this species would be strengthened if levels of the SOR could be determined and found to be altered by the conditions that change life-span. Previous attempts to measure production of the SOR in the rotifer have been unsuccessful because of the large number of animals needed for assay. In this study rotifers were raised successfully in batch cultures which provided sufficient material to determine SOR levels in membrane samples while the animals were raised under various life modification conditions.

The mechanism of SOR production in this model was also examined. The experiments included determination of ATP-dependent Ca^{2+} uptake and measurement of phospholipase A_2 activity and the activities of free radical scavenging enzymes over the life-span of the rotifer. Also, the activities of two lysosomal enzymes were measured to see if they undergo changes during aging.

METHODS

Animals

The assays in this study were performed in whole homogenates and in membrane samples prepared from the rotifers. To obtain sufficient material, the animals were raised in batch culture by adding 250 young (0.25 day old) rotifers to Erlenmeyer flasks containing 1 liter of cerophyll medium with *Paramecium caudatum* as the food source [3,5]. Ca^{2+} was not added to the medium. During culture, the Paramecia in the medium were counted 4 times daily and added when necessary to ensure a concentration of at least 800 Paramecia/ml to prevent diet restriction which increases rotifer life-span [3]. Except where indicated, cultures were maintained at control conditions of 19°C and a 12 h light, 12 h dark cycle. Within 1 to 2 weeks batch cultures contained 3,000–4,000 rotifers.

Determination of Age and Life-Span

Batch cultures contain rotifers of various age. Selection for the different age groups was made on the basis of body length and width, both of which increase linearly over the life-span of this organism [6]. In addition, care was taken to avoid selection of rotifers with young offspring which are usually born at 2.5 days of age. For the biochemical studies, animals were removed from batch culture, measured individually under a dissecting microscope equipped with a micrometer, and placed into the different age groups for preparation of homogenate or membrane samples. When life-span was measured, 24 representative animals from each age group were removed from batch culture and were placed into a well (9.6 cm^2) of a 6-well plate. They were maintained at a similar population density and treatment condition as in the batch culture to verify life-span. Culture wells were viewed every 2 h to determine the time of death.

Life Modification Treatments

Except for the vitamin E and the UV-treated groups, each life modification treatment was administered to rotifers at the start of the batch culture so that the animals were selected from offspring born into one of the various life modifying conditions. The young were removed at 0.25 days of age and either examined immediately for biochemical changes or transferred to another batch culture for further treatment and aging before examination of the various biochemical parameters. For the vitamin E group, the young were born to adult rotifers that were exposed to vitamin E (20 μg/ml of *d*-α-tocopherol, Corvitol F-1000, Henkel Corporation, MN) for 24 h before removal of the young. Treatment of the offspring with this vitamin continued after separation. Diet restricted rotifers were raised in medium in which the Paramecia population was carefully regulated to an estimated 100 per ml. For the temperature study, cultures were grown at 15, 19, or 27°C. For rotifers raised in the dark, the animals were cultured for 20 generations (2.5 day per generation) in complete darkness before samples were collected for assay. Aliquots were removed four times a day and brought into the light for determining Paramecia concentration and discarded. In the UV treatment group, 500, 0.25 day old rotifers were removed from batch culture and treated with UV radiation. They were placed in 300 μl of distilled H_2O to prevent desiccation and exposed to UV radiation from a 4 W mercury lamp (Atomic Labs. Inc., Berkley CA). The lamp output was 10 J/m^2/s at 14 cm and at a wavelength of 253.7 nm. Length of exposure time was used to determine the dose of 300 J/m^2 [7]. Controls for this group were exposed with 4 W incandescent light for the same duration. The homogenates and the membrane samples were prepared immediately after exposure for the 0.25 day old group or the animals were maintained in a separate batch culture until 1.5, 3, or 5 days of age for subsequent preparation of older rotifers.

Enzyme Inhibitors

Rotifers were treated with enzyme inhibitors to examine the mechanism of SOR formation. Batch cultures were exposed to 20 mM bromophenacyl bromide, which inhibits phospholipase A_2, or to 250 μM indomethacin, which inhibits cyclooxygenase, for 12 h immediately before removal for membrane preparation. The inhibitor concentrations were selected from dose–response curves (life-span vs. concentration). Exposure to the above concentration of bromophenacyl bromide or indomethacin for 12 h did not affect rotifer life-span.

Homogenate Samples

The samples were prepared by removing approximately 500 rotifers from batch culture. After determination of age, they were homogenized with 5 strokes of a motor-driven Teflon-glass homogenizer in a tube with 300 μl of 20 mM EPPS (*N*-[2-hydroxyethyl]-piperazine-*N*′-3-propanesulfonic acid) buffer, pH 6.8, at 4°C. Samples were centrifuged at 800*g* for 15 min to remove large particles and the supernatant was used for the lipid peroxide and for enzyme assays. Protein levels in homogenates were determined by the procedure of Sorensen and Brodbeck [8] using bovine serum albumin as the standard, and phosphate content was measured as described by Dittmer and Wells [9].

Membrane Samples

Membrane samples were prepared using a modification of the method described by Brunnette and Till [10]; this procedure separates membranes according to surface characteristics. Approximately 1,500 rotifers were collected and their age determined as described above. They were homogenized at 4°C in 20 mM EPPS with 5 strokes of a motor-driven Teflon-glass homogenizer. During preparation, the samples were maintained in a nitrogen atmosphere to minimize oxidation. The homogenate was brought to 0.1 μM with $ZnCl_2$ and centrifuged for 15 min at 2,500*g* (4°C). The pellet was mixed with 5 ml of dextran (20% w/w) and 4 ml of polyethylene glycol (30% w/w), which were prepared as described by Sawada and Carlson [11]. Each sample was split into 2 equal volumes of 4.5 ml and centrifuged for 15 min at 9,000*g*. After centrifugation, membranes located at the interfaced were removed, combined, and diluted with five volumes of 20 mM EPPS buffer. The solution was mixed and centrifuged at 12,000*g* for 20 min to collect the membrane pellet. The protein content was determined as described above.

Marker Enzymes

Membrane samples were assayed for marker enzymes to determine the source and enrichment of the membrane samples. Ouabain-sensitive K^+–Na^+-ATPase was used as the plasma membrane marker [12]; the activity was expressed as μg of phosphate produced per mg protein per hour. NADH-cytochrome *c* reductase activity served as the endoplasmic reticulum marker [13] and was expressed as μmol cytochrome *c* reduced per mg protein per hour. Succinate dehydrogenase activity [14] was used as the mitochondrial marker and was expressed as μmol *p*-iodonitrotetrazolium violet reduced per mg protein per hour. Enrichment of the enzyme was expressed as the specific activity of membrane sample divided by specific activity of the homogenate.

ESR Spectroscopy

Levels of SOR in membrane samples were determined by measuring the height of the ESR signal that was produced when freshly prepared Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) reacted with SOR [15,16]. Tiron reacts with SOR to generate a 4 peak spectrum [17]. The signal was measured at 5 min, which corresponds to the time when a steady state was reached [18]. The basic procedure followed that used previously in our laboratory [11,19] except that each sample contained 150 μg of membrane protein and was placed in a flat cell which was positioned in the sample holder of a Varian E-12 spectrometer. The spectrometer was set at a field setting of 3365 G. The microwave frequency was 9.14 GHz, the microwave power was 10 mW, and the modulation amplitude was 1 G. The time constant was 1 s and the total time of the scan was 4 min. The spectrometer was calibrated using the xanthine oxidase/xanthine SOR generating system [20].

Lipid Peroxide Measurement

Lipid peroxide levels in homogenates of rotifers were determined by the thiobarbituric acid test [21] as modified by Sawada and Carlson [5]. The results are expressed as ng of standard malonaldehyde (bis[dimethyl acetal], Aldrich, Milwaukee, WI) per mg of protein.

Free Radical Scavening Enzymes

The activities of free radical scavenging enzymes were determined in rotifer homogenates (100–200 μg protein per sample). Superoxide dismutase levels were determined by the method of Crapo et al. [22]. It is based on the amount of enzyme required for 50% inhibition of cytochrome *c* reduction. Catalase activity was estimated by a spectrophotometric method [23] which measures the decomposition of hydrogen peroxide. Glutathione peroxidase activity was determined by the procedure of Maral et al. [24]. This method measures the oxidation of NADPH.

Lysosomal Enzymes

The activities of α-mannosidase were measured in rotifer homogenates (150–250 μg protein) using the spectrophotometric method described by Barrett [25]. The activities of β-glucuronidase in homogenates were determined by the procedure described in Rosenfeld et al. [26].

Membrane Uptake of Ca^{2+}

ATP-dependent Ca^{2+} uptake was measured in membrane samples of rotifers at different ages using the method of Minami and Penniston [27] to determine if there is an increase with age. The effects of vitamin E or UV radiation on Ca^{2+} uptake were also investigated.

Phospholipase A_2 Activity

Membrane-bound phospholipase A_2 activity was determined by the method of Petkova et al. [28]. Membrane samples containing 120 μg of protein were used, and they were suspended in 5 mM Tris–HCl buffer (pH 8.5) containing 5 mM $CaCl_2$. The specific activity was calculated as nmol of fatty acid released per min per mg of membrane protein.

Statistical Analyses

Significance was measured by one-way ANOVA followed by the post hoc Tukey test for comparison between the age groups or Duncan's multiple range test for comparison within each experimental treatment. The calculations were carried out using the LCS Stat-Lab V2.20 (LCS Inc., Montreal, Quebec).

RESULTS

Marker Enzymes

The activities of marker enzymes were measured to determine the enrichment and purity of the plasma membrane sample. Table I shows that there is nearly a 10-fold enrichment in the plasma membrane marker and that there is also evidence of membranes from the endoplasmic reticulum in the sample.

Effects of Life Modification

The initial experiments were performed to determine if various life-span modifying conditions also resulted in a corresponding change in the SOR levels in membrane samples. The results of this study appear in Tables II and III. SOR levels were higher in older rotifers than in younger animals ($P < 0.05$), and a significant difference was seen due to the effects of the various life modification conditions when the results were

TABLE I. Marker Enzyme Activities of Rotifer Membrane Samples*

	Succinate dehydrogenase	Cytochrome *c* reductase	Na^+–K^+-ATPase
Specific activity	0.32 ± 0.25	1.39 ± 0.41	1.36 ± 0.64
Enrichment	0.37 ± 0.41	3.45 ± 0.72	9.86 ± 0.58

*The specific activity of succinate dehydrogenase (mitochondrial marker) represents the μmol of *p*-iodonitrotetrazolium violet reduced/mg protein/h. The specific activity of cytochrome *c* reductase (endoplasmic reticulum marker) refers to the μmol of cytochrome *c* reduced/mg protein/h. The specific activity of Na^+–K^+-ATPase (plasma membrane marker) represents the μg of PO_4 produced/mg protein/h. Enrichment equals the specific activity of the membrane sample/specific activity of homogenate. Each value represents the mean ± SE of five replicates. The average membrane yield was 369 ± 40 μg of protein (mean ± SE).

TABLE II. Life-Span, SOR Levels, and Malonaldehyde Levels in 0.25 Day Old Rotifers†

Life modification condition	Remaining life-span (days)	SOR (cm)	Malonaldehyde (ng)
15°C	7.3 ± 0.3*	1.7 ± 0.2**	1.0 ± 0.5
19°C (control)	5.4 ± 1.0	2.5 ± 0.2	1.0 ± 0.8
Vitamin E	6.9 ± 1.2*	1.5 ± 0.2**	1.3 ± 1.3
Complete darkness	6.5 ± 0.9*	1.7 ± 0.2**	0.7 ± 0.7
Diet restriction	6.6 ± 0.7*	1.7 ± 0.2**	0.9 ± 0.9
27°C	4.7 ± 0.8*	3.6 ± 0.2**	1.6 ± 1.4
UV	4.3 ± 1.0*	3.8 ± 0.4**	1.7 ± 1.7

†Each value represents the mean ± SE. Sample size is 24 rotifers for the life-span groups and 4 preparations for each group in the SOR and malonaldehyde study. Young rotifers were removed from batch culture at 0.25 days of age. The remaining life-span corresponds to the number of days of survival after removal from batch culture. Significance was examined by comparison with 19°C (controls). The levels of SOR correspond to the height (cm) of the ESR signal formed as TIRON reacts with SOR. Significance was determined by comparison with 19°C (controls).
*$P < 0.01$.
**$P < 0.05$.

examined by two-way ANOVA ($P < 0.01$). In membrane samples from young rotifers (Table II), each life-span modification factor that extended life-span significantly lowered SOR levels, and each factor that shortened life-span significantly increased SOR levels. A similar trend appeared under most of the life modification conditions in the older animals (Table III). The correlation coefficient between life-span and SOR levels was 0.89 for young and 0.95 for old rotifers. A similar significant relationship between life modification condition and malonaldehyde levels was found in homogenates prepared from old rotifers. Young rotifers, however, contain much less malonaldehyde ($P < 0.01$) than older animals, and there was no noticeable effect of treatment at 0.25 days of age.

Inhibition of ESR Signal

In order to examine the mechanism of SOR formation, rotifers were exposed to bromophenacyl bromide or indomethacin, which inhibits the synthesis of prostaglandins. The ESR signal was significantly suppressed when each inhibitor was added to the batch culture for 12 h immediately before membrane preparation (Table IV). In

TABLE III. Life-Span, SOR Levels, and Malonaldehyde Levels in 5.0 Day Old Rotifers†

Life modification	Remaining life-span (days)	SOR (cm)	Malonaldehyde (ng)
15°C	1.4 ± 0.6	2.4 ± 0.3**	39.2 ± 4.3**
19°C (control)	0.9 ± 0.6	3.9 ± 0.3	53.2 ± 4.7
Vitamin E	1.6 ± 0.8	2.3 ± 0.0**	37.7 ± 3.5**
Complete darkness	1.8 ± 0.6	2.9 ± 0.4**	38.3 ± 1.9**
Diet restriction	1.6 ± 0.8	3.2 ± 0.3	39.5 ± 2.5**
27°C	0.0 ± 0.0*	4.5 ± 0.3	74.7 ± 4.4*
UV	0.0 ± 0.0*	5.0 ± 0.5**	76.0 ± 4.2*

†Each value represents the mean ± SE. Sample size is 24 rotifers for the life-span groups and four preparations for each group in the SOR and malonaldehyde study. Old rotifers were removed from batch culture at 5.0 days of age. The remaining life-span corresponds to the number of days of survival after removal from batch culture. Significance was examined by comparison with 19°C (controls). The levels of SOR correspond to the height (cm) of the ESR signal formed as TIRON reacts with SOR. Significance was determined by comparison with 19°C (controls).
*$P < 0.01$.
**$P < 0.05$.

TABLE IV. Inhibition of SOR Formation in Membrane Samples From 5 Day Old Rotifers†

Treatment	ESR signal strength (cm)
Control	3.6 ± 0.5[a]
Bromophenacyl bromide	2.3 ± 0.4[b,*]
Indomethacin	2.1 ± 0.2[b,*]
100°C	0.0[a,**]
Superoxide dismutase	0.20 ± 0.2[a,**]

†For treatment with bromophenacyl bromide, rotifers were exposed to 20 mM for 12 h in batch culture immediately before removal for ESR. Indomethacin treated rotifers were exposed to 250 μM for 12 h in batch culture immediately before removal for ESR. In the remaining groups, membrane samples were treated by heating to 100°C for 20 min or by exposing to 500 μg of SOD for 15 min immediately before ESR.
[a]Five replicates.
[b]Three replicates.
*$P < 0.05$.
**$P < 0.01$.

addition, heating membrane samples from 5 day old rotifers to 100°C for 20 min or adding superoxide dismutase for 15 min prior to measurement blocked the ESR signal.

Ca^{2+} Uptake in Membrane Samples

A study was performed to determine if there were changes in Ca^{2+} uptake in aging rotifers and if this alteration was sensitive to life modification conditions. There was a significant increase in uptake by day 3 in controls ($P < 0.01$) as well as in the UV irradiated ($P < 0.01$) and vitamin E ($P < 0.05$) groups (Fig. 1). Furthermore, Ca^{2+} uptake was significantly higher in the UV-treated group at 3 and 5 days of age ($P < 0.01$) and significantly less in the vitamin E group at 3 and 5 days of age ($P < 0.05$) than in the controls.

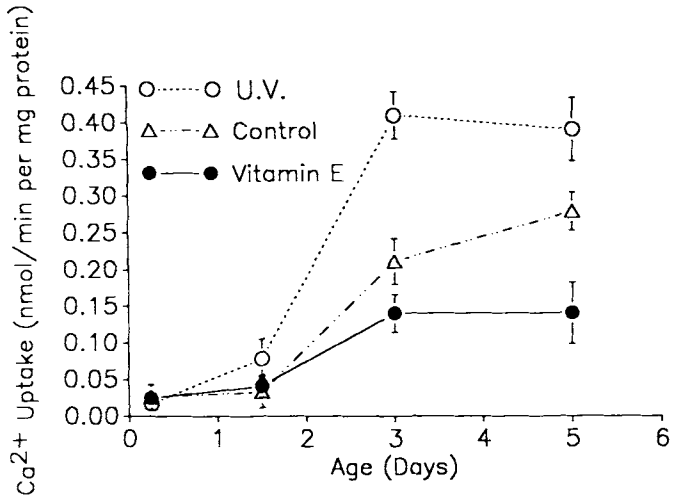

Fig. 1. Ca^{2+} uptake in membrane samples. Rotifers were treated with UV (300 J/m^2) radiation at 0.25 days or exposed to 20 μg/ml of *d*-α-tocopherol in batch cultures. Ca^{2+} uptake was measured at various ages. Each group was raised at 19°C in a 12 h light, 12 h dark cycle in cerophyll medium containing Paramecia as the food source. A significant increase in Ca^{2+} uptake was seen at day 3 and 5 in each group (controls and UV, $P < 0.01$; vitamin E, $P < 0.05$). Also on days 3 and 5 Ca^{2+} uptake was significantly greater in the UV group ($P < 0.01$) and significantly less in the vitamin E group ($P < 0.05$) than in the controls. Each point represents the mean ± SE of four experiments.

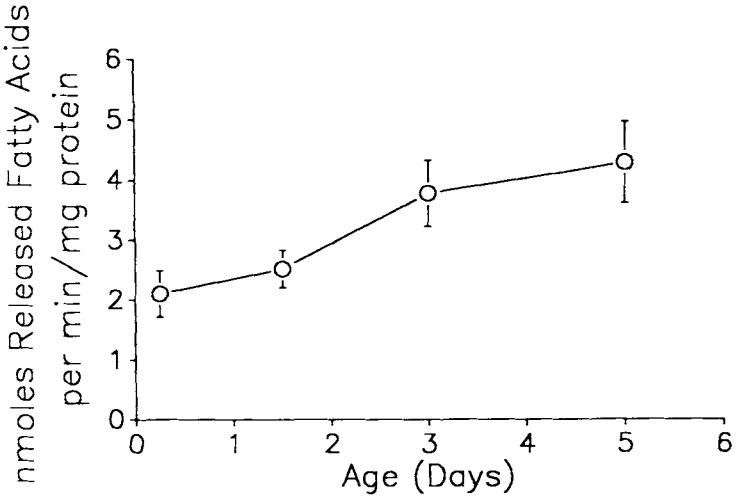

Fig. 2. Phospholipase A_2 activity in membrane samples. Samples were prepared from rotifers at different ages and incubated with 170 nmol 1-acyl-2-[^{14}C]linoleoyly-*sn*-glycerophospho-ethanolamine for 15 min in Tris–HCl buffer with 5 mM $CaCl_2$, pH 8.5. After incubation, samples were extracted with chloroform: methanol (2:1), chromatographed by TLC, and counted in a liquid scintillation counter. A significant increase in activity occurred on days 3 and 5 ($P < 0.05$). Each point represents the mean ± SE of four experiments.

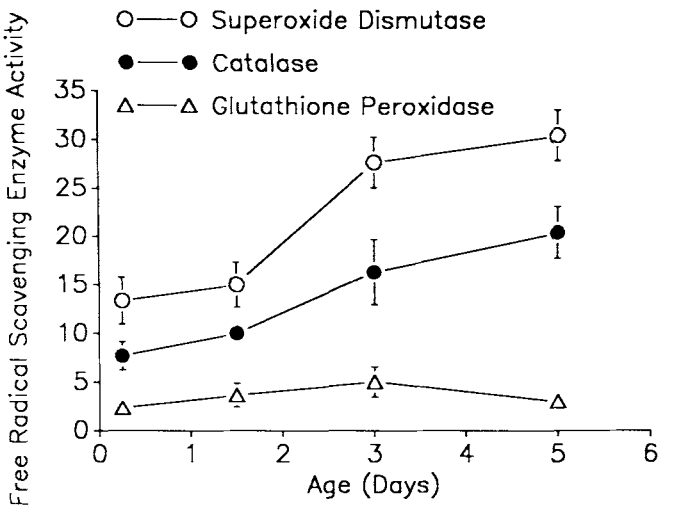

Fig. 3. Free radical scavenging enzyme activity of homogenate samples. The reaction mixture for superoxide dismutase contained 2.1 ml of 50 mM (pH 7.8) potassium phosphate buffer which included 0.3 ml of 0.1 mM cytochrome *c*, 0.3 ml of 0.5 mM xanthine, and 0.3 ml of 1 mM KCN. Superoxide dismutase activity is based on the increase in absorbance at 550 nm and it is expressed as units of enzyme per mg of protein. The reaction mixture for catalase contained 0.01 M phosphate buffer pH 7.0, 2 mM H_2O_2, and 100–200 μg of protein in a total volume of 1 ml. Catalase activity corresponds to the change in absorbance at 245 nm per minute per mg of homogenate protein. The reaction mixture for the glutathione peroxidase contained 0.2 mM NADPH, 1 mM reduced glutathione, 1 mM *t*-butylhydroperoxide, 3 IU of yeast glutathione reductase, and 100–200 μg of protein in 1.5 ml of 5×10^{-2} M phosphate buffer at pH 7.0. Glutathione peroxidase activity corresponds to the μmol of NADPH oxidized (as measured at 340 nm) per minute per mg of homogenate protein. A significant increase in the activity of superoxide dismutase and catalase was seen by day 3 ($P < 0.05$). Each point represents the mean ± SE of four experiments.

Changes in Phospholipase A_2

Figure 2 shows the phospholipase A_2 activity in membrane samples from rotifers of various ages. A significant increase in activity was seen in rotifers at 3 and 5 days of age ($P < 0.05$).

Free Radical Scavenging Enzymes

The activities of superoxide dismutase, catalase, and glutathione peroxidase were measured in homogenates of rotifers to determine if there were changes with age (Fig. 3). Elevated activities of superoxide dismutase and catalase were seen at 3 and 5 days of age with respect to the 0.25 day old rotifer ($P < 0.05$). However, there was no significant alteration in glutathione peroxidase activity during the life-span of rotifers ($P > 0.50$).

Lysosomal Enzyme Alteration With Age

Aging may result in changes to lysosomes. To study this possibility, two lysosomal enzymes, α-mannosidase and β-glucuronidase, were examined in homogenates prepared from rotifers at different ages (Fig. 4). The activity of α-mannosidase increased over the life time but the rise did not become significant until 5 days of age ($P < 0.05$). However, no significant age difference in activity for β-glucuronidase was detected ($P > 0.05$).

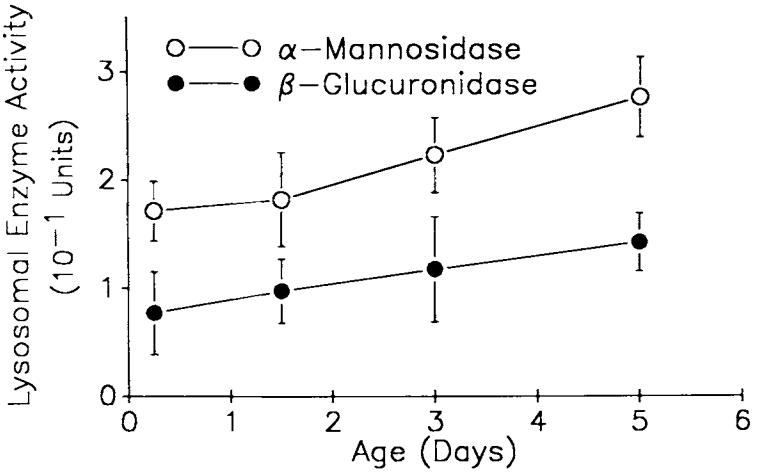

Fig. 4. Lysosome enzyme activity. Activities of α-mannosidase and β-glucuronidase were measured in rotifer homogenates during the life-span. Activities of the enzymes are expressed in enzyme units which represent μmol of substrate transformed per minute per mg of homogenate protein. The assay for α-mannosidase contained 0.5 ml of 0.3 M sodium citrate–citric acid buffer with 3 mM $ZnCl_2$, pH 4.6, 0.5 ml of 1.5 mM 4-methylumbelliferyl glycoside, and 150–250 μg of protein. The absorbance was measured at 360 nm. The assay for β-glucuronidase contained 1 ml of 0.2 M sodium acetate buffer, pH 4.5, 0.1% BSA, 10 mM *P*-nitrophenol β-D-gucosiduronic acid, and 150–250 μg of protein. The absorbance was read at 400 nm. There was a significant increase in α-mannosidase activity on day 5 ($P < 0.05$).

DISCUSSION

The results of this study indicate that SOR levels are detectable in membrane samples of rotifers and that there is a measurable increase with age. Increased concentrations of SOR have been found in mitochondrial samples from the brain and the heart of aging rats [19,29], in plasma membranes from regressing rat corpora lutea [11], and in microsomes prepared from senescing plants [18].

In the present study, SOR levels were found to be sensitive to the different experimental conditions that alter life-span in the rotifer. Some of these conditions appear to work by altering metabolism. For example, diet restriction, absence of light, and temperature changes may alter SOR production by changing the general metabolic rate [1,30] as well as the activity of SOR generating enzymes [31]. Vitamin E, however, works differently, since it is a free radical scavenger [32,33]. In contrast, UV treatment induces the formation of free radicals [4,34]. Elevated production of SOR could account for the shortened life-span that we observed in the rotifer. SOR generates formation of more toxic free radicals (e.g., hydroxyl radicals), which cause damage to biological molecules. Numerous cellular sites of oxyradical damage have been noted (e.g., DNA, collagen, enzymes). The list includes cellular membranes where free radicals stimulate peroxidation of polyunsaturated fatty acids [2,35]. In the present study, we observed an increase in SOR levels prior to a rise in malonaldehyde (Table II). In addition, in 5 day old rotifer there was a close correspondence between alterations in SOR production and changes in the generation of thiobarbituric acid reactive material. The life modifying conditions that decreased levels of this free radical also lowered lipid peroxide production, whereas those conditions that raised

SOR levels also favoured lipid peroxide production. These changes indicated that increased SOR formation may be involved in mediating the molecular and cellular changes responsible for aging in this species.

Several interesting changes occurred in the rotifer on day 3 when there was a pronounced increase in Ca^{2+} uptake and a significant rise in phospholipase A_2 activity in membrane samples and an elevation in activity of superoxide dismutase and catalase in homogenate samples. The increase in Ca^{2+} uptake was sensitive to UV radiation and vitamin E treatment. Normally homeostatic processes work to maintain low intracellular Ca^{2+} concentration to prevent toxicity [36]. However, there is evidence that the balance of free and bound Ca^{2+} is upset in aging cells as bound levels of this divalent cation have been found to rise in older cells [37]. In the present study, the elevation in Ca^{2+} uptake corresponded temporally with the greatest increase in phospholipase A_2 activity. Ca^{2+} acts as an intracellular messenger to stimulate many enzymes including phospholipase A_2 [38]. Phospholipase A_2 causes deesterification of phospholipids resulting in free fatty acid release and membrane perturbation [39]. Higher levels of activity of this lipolytic enzyme have been reported in liver samples from aging rats [28]. Elevated levels of arachidonic acid may be responsible for the rise in SOR observed in older rotifers in the present study. Arachidonic acid stimulates production of this free radical by activating NADPH oxidase in the plasma membrane of neutrophils [40], and recently we observed an increase in phospholipase A_2 activity [41] and SOR production in rat ovarian tissue during corpus luteum degeneration at the end of the reproductive cycle [11]. Evidence that this pathway may be active in aging rotifers was obtained in the present study. Addition to batch cultures of bromophenacyl bromide, which inhibits phospholipase A_2, and indomethacin, which inhibits arachidonic acid metabolism via the cyclooxygenase pathway [42], lowered SOR production in older rotifers. These results indicate the possibility that rotifers and mammals may possess similar SOR generating systems, and they raise the question of a similar pathway being involved in the aging process in mammals.

Enzymatic defences work to remove SOR and hydrogen peroxide to protect cells from the damaging effects of these compounds. One explanation for the increase in SOR with age in the rotifer is that the elevation could be due to a loss in activity of free radical scavenging enzyme activity. Superoxide dismutase converts SOR to hydrogen peroxide [43], which is metabolized by catalase or glutathione peroxidase [44]. Species difference in activity of these enzymes may account for the dissimilarity in life-span among various organisms [45]. Longitudinal studies in which the activities of scavenging enzymes were measured indicate that the general trend seems to be a reduction with age. Superoxide dismutase levels were found to decrease with age in the rat liver [46] and in the rat nervous system [47]. In addition, catalase activity was observed to be lower in the later stages of life in *Drosophila* [48]. Also a decrease in activity of superoxide dismutase and catalase was reported in aging houseflies [49]. However, in rotifers we found a significant increase in superoxide dismutase and catalase activity with age. This elevation may represent an attempt by this organism to protect itself from the effect of increasing levels of SOR.

One of the intracellular sites affected by SOR may be the lysosomes, which contain enzymes that digest organelles and macromolecules. Their function is important in cellular repair [50]. An increase in levels of SOR in rotifers may inflict damage to intracellular membranes and in the case of lysosomes may cause the release of

hydrolytic enzymes. Such a response could account for the elevation in α-mannosidase activity that we observed in homogenates prepared from older rotifers. Increases in activity of lysosomal enzymes in senescing cells in vitro have been described previously [51].

The sequence of changes observed in this study indicates that SOR could mediate the degenerative process in aging rotifers. An important early event seems to be an increase in intracellular Ca^{2+} binding which has been reported in other aging systems [37]. This change is associated with the elevation of phospholipase A_2 activity observed on day 3. The resulting phospholipid deesterification generates arachidonic acid that may stimulate SOR formation [11,40] and lipid peroxidation and may contribute to membrane breakdown and organelle changes. Our findings indicate that the SOR increase is not associated with a decrease in activity of superoxide dismutase in the rotifer. The fact that SOR and lipid peroxides are closely tied to environmental conditions that alter life-span suggests that increased production of SOR may play an important role in mediating the biochemical changes that occur during cellular breakdown in the aging rotifer.

ACKNOWLEDGMENTS

The authors would like to thank Henkel Corporation, Minneapolis, MN, for the vitamin E, and LCS Inc, Montreal, Canada, for the LCS STAT-LAB V2.20 Software package and for the assembler computer programs for instrument interface and data acquisition. This work was supported by the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

1. Harman D: Proc Natl Acad Sci USA 78:7124–7128, 1981.
2. Slater TF: "Free Radical Mechanisms in Tissue Injury." London: Pion limited, 1972, pp 21–33.
3. Sawada M, Carlson JC: J Gerontol 42:451–456, 1987.
4. Sawada M, Carlson JC, Enesco HE: Arch Gerontol Geriat 10:27–36, 1990.
5. Sawada M, Carlson JC: Exp Gerontol 20:179–186, 1985.
6. Sawada M, Enesco HE: Exp Gerontol 19:179–183, 1984.
7. Sawada M, Enesco HE: Exp Gerontol 19:289–296, 1984.
8. Sorensen K, Brodbeck U: Experientia 42:161–162, 1986.
9. Dittmer JM, Wells MA: Methods Enzymol 14:482–530, 1969.
10. Brunette DM, Till JE: J Membrane Biol 5:215–224, 1971.
11. Sawada M, Carlson JC: Can J Physiol Pharmacol 67:465–471, 1989.
12. Cole CH, Waddell RW: J Clin Endocrinol Metab 42:1056–1063, 1976.
13. Sottocasa GL, Kuylenstierna B, Ernster L, Bergstrand A: J Cell Biol 32:415–438, 1967.
14. Pennington RJ: Biochem J 80:649–654, 1961.
15. Greenstock CL, Miller RW: Biochim Biophys Acta 396:11–16, 1975.
16. Miller RW, MacDowell FDH: Biochim Biophys Acta 387:176–187, 1975.
17. McRae DG, Baker JE, Thompson JE: Plant Cell Physiol 23:375–383, 1982.
18. Leshem YY, Sridhara S, Thompson JE: Plant Physiol 75:329–335, 1984.
19. Sawada M, Carlson JC: Mech Aging Dev 41:125–137, 1987.
20. Beauchamp C, Fridovich I: Anal Biochem 44:276–287, 1971.
21. Uchiyama M, Mihara M: Anal Biochem 86:271–278, 1978.
22. Crapo JD, McCord JM, Fridovich I: Methods Enzymol 53:382–393, 1978.
23. Chance B, Maehly AC: Methods Enzymol 2:764–775, 1955.
24. Maral J, Puget K, Michelson AM: Biochem Biophys Res Commun 77:1525–1535, 1977.

25. Barrett AJ: In Dingle JT (ed): "Lysosomes a Laboratory Handbook." Amsterdam: North Holland Publishing Co, 1972, pp 46–135.
26. Rosenfeld MG, Kreibich G, Sabatini DD, Kato K: Methods Enzymol 96:764–777, 1983.
27. Minami J, Penniston JT: Biochem J 242:889–894, 1987.
28. Petkova DH, Momchilova AB, Koumanov KS: Exp Gerontol 21:187–193, 1986.
29. Nohl H, Hegner D: Eur J Biochem 82:563–567, 1978.
30. Sohal RS, Allen RG: In Woodhead AD (ed): "Molecular Biology of Aging." New York: Plenum Press, 1984, pp 75–104.
31. Cohen HJ, Chovaniec ME: J Clin Invest 61:1081–1087, 1987.
32. Ozawa T, Hanaki A, Matsumoto S, Matsuo M: Biochim Biophys Acta 531:72–78, 1988.
33. Burton GH, Ingold KV: J Am Chem Soc 103:6472–6479, 1981.
34. Black HS: Photochem Photobiol 16:213–221, 1987.
35. Kellogg EW III, Fridovich I: J Biol Chem 250:8812–8817, 1975.
36. Hochachka PW: Science 231:234–241, 1986.
37. Peterson C, Goldman JE: Proc Natl Acad Sci USA 83:2758–2762, 1986.
38. Cheung WY: Science 207:19–27, 1980.
39. Stocker R, Richter C: FEBS Lett 147:243–246, 1982.
40. Clark RA, Leidal KG, Pearson DW, Nauseef, WM: J Biol Chem 262:4065–4074, 1987.
41. Riley JCM, Carlson JC: Endocrinology 121:776–781, 1987.
42. Vane JR: Nature (London) 231:232–235, 1971.
43. Fridovich I: Annu Rev Biochem 44:147–159, 1975.
44. Leibovitz BE, Siegel BV: J Gerontol 35:45–56, 1980.
45. Cutler RG: In Woodhead AD (eds): "Molecular Biology of Aging." New York: Plenum Press, 1984, pp 15–73.
46. Reiss U, Gershon D: Eur J Biochem 63:617–623, 1976.
47. Vanella A, Geremia E, D'Urso G, Tiriolo P, Di Silvestro I, Grimaldi R, Pinturo R: Gerontology 28:108–113, 1982.
48. Massie HR, Baird MB: Mech Aging Dev 5:39–43, 1976.
49. Sohal RS, Farmer KJ, Allen RG, Cohen NR: Mech Aging Dev 26:75–81, 1983.
50. Tappel AL: Fed Proc 24:73–78, 1965.
51. Milisauskas V, Rose NR: Exp Cell Res 81:279–284, 1973.