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Impact Factor: 3.02 · DOI: 10.1006/abbi.1995.1248 · Source: PubMed

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Aging of the Liver: Proteolysis of Oxidatively Modified Glutamine Synthetase

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Received January 12, 1995, and in revised form January 27, 1995

During aging cells accumulate altered forms of proteins, notably oxidatively modified proteins. The multicatalytic protease selectively degrades oxidized proteins, suggesting that the age-related accumulation of oxidized proteins might be a consequence of decreased activity of this protease. The protease activity of liver homogenates was assayed with an improved fluorimetric method, using oxidatively modified glutamine synthetase as substrate. Application of this assay to extracts from liver of Fischer 344 rats from both Japan and the United States demonstrated a marked preference for the oxidized substrate, as expected. Extracts from animals ages 8 to 26 months maintaine both total proteolytic activity and the ability to distinguish between native and oxidized substrates. Oxidatively modified hepatocyte extracts were also employed as substrate, and older animals again maintained proteolytic activity. The multicatalytic protease was purified from liver of young and old rats, and the specific activity of the preparations were comparable when assayed with oxidatively modified glutamine synthetase. We conclude that the intrinsic neutral or alkaline proteolytic activity of rat liver is maintained during aging.

The morphological and functional changes which occur during aging have been described over centuries, but the mechanisms underlying those changes are not understood. Experimental evidence published in recent years has focused attention on the role of free radical-mediated reactions in the aging process, a concept initially proposed by Harman (1, 2). An increase in free radical production during aging of *Drosophila* has been reported (3), and it is well established that free radicals react with a variety of cellular components, often in a self-perpetuating cascade reaction. Oxidation of lipids has long been appreciated, and such reactions may disrupt function directly or give rise to toxic products which act at a distance (4). Reactions with nucleic acids may give rise to mutations which impair function or regulation (5). More recent investigations have focused on the possibility that oxidation of proteins is important during aging, especially that mediated by metal-catalyzed oxidation (6). During aging, the activities of many enzymes change, often decreasing (7, 8). Comparison of a list of enzymes susceptible to metal-catalyzed oxidation revealed a striking overlap with a list of enzymes whose specific activities decline during aging (9). This led to the suggestion that accumulation of oxidatively damaged proteins contribute to the changes of aging (9, 10). An increase in the content of oxidized proteins during aging has been reported for human fibroblasts, human lens, rat liver, gerbil brain, and whole fly (11–15). Functional defects have been correlated with this accumulation (14, 16).

An increase in the steady-state level of oxidatively modified proteins could result from an increased rate of oxidation or from a decreased rate of degradation of the damaged proteins. At present there are no methods available for measuring the rate of protein oxidation, but a decrease in activity of neutral or alkaline proteases toward oxidized proteins was described for rat liver (13) and gerbil brain during aging (14). Agarwal and Sohal recently reported a lack of change in protease activity with age in the housefly, using X-irradiated albumin as substrate at pH 8.0 (17). With the same assay, they found little change in protease in homogenates of brain or heart from the rat, but did confirm a loss of almost 50% in protease activity from liver at 23 months compared to 3 months of age. The activity measured in each of these cited studies may have been that of the multicatalytic protease, which is known to

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0003-9861/95 $6.00

discriminate between oxidized and native proteins (18). The experiments reported in this paper were instituted with the purpose of investigating the mechanisms which cause the age-related increase in oxidatively modified proteins. Rat liver maintained its ability to degrade both oxidatively modified liver homogentates and oxidatively modified glutamine synthetase. Multicatalytic protease purified from young and old rat livers exhibited similar apparent K_m and V_{max} toward oxidatively modified glutamine synthetase.

MATERIALS AND METHODS

Materials and Enzymes. Fluorescamine was obtained from Sigma (St. Louis, MO) and a stock solution of 0.30 mg/ml was prepared in acetone. A positive displacement pipette delivered aliquots during the assay (Microman from Rainin, Woburn, MA). For all enzymes assayed, a unit is defined as that activity which produces product at the rate of 1 μmol/min. Error bars (standard error of the mean) are shown in the figures when at least three separate animals were assayed. Glutamine synthetase was prepared and oxidatively modified for 8 h as described (19). Liver extracts were assayed for glutamine synthetase activity with the γ-glutamyl transferase assay at pH 7.57 (20). Glucose-6-phosphate dehydrogenase activity was assayed at 25°C, after removal of low-molecular-weight substances which interfere with the assay. The liver supernatant was diluted fivefold in 10 mM Mops,[3] 150 mM sodium chloride, pH 7.4, and 1 ml was chromatographed on a Sephadex G25M PD10 column (Pharmacia, Piscataway, NJ). The assay was initiated by addition of 50 μl sample to 950 μl of buffer containing 0.5 mM $NADP^+$ and 5.0 mM 6-phosphogluconate, either with or without 5.0 mM glucose-6-phosphate. Production of NADPH was followed at 340 nm in a Hewlett-Packard Model 8452A diode array spectrophotometer (Palo Alto, CA). Glucose-6-phosphate dehydrogenase activity was calculated as the difference in rate of NADPH production in the presence and absence of glucose-6-phosphate. Enzyme activity was linear with time under these assay conditions.

The multicatalytic protease was purified from the liver of 8- and 24-month-old male Fischer 344 rats and assayed as described (21), except that the starting material here was freshly removed liver rather than acetone powder. Livers were disrupted with a Potter-Elvehjem homogenizer in 20 mM Hepes, 1 mM 2-mercaptoethanol, 0.1 mM EDTA, pH 8.0. After centrifugation for 2 h at 25,000*g*, the supernatant was processed as described (21).

Liver homogenates were oxidized to provide substrate for measurement of proteolysis as described for oxidation of glutamine synthetase[4] (19, 22). Specifically, homogenates at 10 mg/ml protein were incubated at 37°C for 24 h in a solution of 200 μM $FeCl_3$, 50 mM ascorbate, 100 mM Hepes, 100 mM KCl, 10 mM $MgCl_2$, pH 7.4. The cloudy solution was centrifuged at 12,000*g* for 20 min, yielding a clear supernatant which was then dialyzed against at least 100 vol of the same solution without iron and ascorbate. The dialysis solution was changed four times, with 1 mM EDTA included in the first two solutions to assist in removal of the iron. The preparation was filtered through a 0.22-μm filter (Millex-GV, Millipore, Bedford, MA), and aliquots were stored at −20°C.

Preparation of liver homogenates. Liver slices were from Fischer 344 rats raised in the specific pathogen-free colony at the aging farm of the Tokyo Metropolitan Institute of Aging. Lighting (8:00 AM to 8:00 PM), temperature (23 ± 2°C), and humidity (50%) were controlled. Other details of the husbandry and survival data have been published (23). The median survival time was 28 months for males and 30 months for females, determined by a study of 100 animals of each sex some 15 years ago. While no similar large scale study has been done since then, the survival curve of 35 male rats to 18 months was the same as that observed in the earlier study (24). Animals were brought from the aging farm to a conventional clean facility where they were housed for at least 1 week. Husbandry conditions were the same as at the aging farm except for the use of unilateral filtered air flow instead of specific pathogen-free conditions. Animals were not starved before killing, which was by decapitation between 10:00 and 11:00 AM. Immediately after decapitation, livers were excised and cut into pieces of 1–3*g*, frozen by dry ice, and held at −90°C until shipment to the United States, where they were stored at −70°C until assayed. Seven livers were obtained from animals at each of three ages, 8, 14, and 26 months. Three animals were male and 4 were female.

Male Fischer 344, Fischer 344/Brown Norway hybrid, and Sprague–Dawley rats were also obtained from the National Institute of Aging colonies maintained at Harlan Sprague–Dawley, Inc. (Indianapolis, IN). The animals were fed the NIH-31, Teklad Premier diet and maintained on a cycle of light (6:00 AM to 6:00 PM)/dark (6:00 PM to 6:00 AM). Rats were anesthetized by intraperitoneal injection of sodium pentobarbital, 1 mg/kg, following which the abdomen was opened and the liver excised. It was rinsed in 10 mM Mops, 150 mM NaCl, pH 7.4, blotted on Whatman 1 filter paper, and weighed. Homogenization was in the same buffer (2 ml per g liver) using a Potter-Elvehjem glass homogenizer with a Teflon pestle. Hepatocyte isolations were kindly performed by Dr. P. Starke-Reed as described (13, 25). Protein was determined by either the Coomassie blue binding assay (26) or the bicinchoninic acid method (27) (Pierce, Rockland, IL).

Assay for proteolysis. The earlier studies of protease activity in liver extracts utilized a mixture of proteins as substrates (13). This substrate mixture was prepared by overnight exposure of a hepatocyte homogenate to an oxidizing system consisting of iron and ascorbate.[5] The preparation has residual endogenous protease activity which gives rise to a background activity that is relatively high and somewhat variable[5] (see Results). This difficulty has now been obviated by substituting oxidatively modified bacterial glutamine synthetase as the substrate (22, 28). Coupled with an HPLC fluorimetric detector, the modified assay allowed determination of the rates of proteolytic activity in extracts from rats of varying age.

The method is based on the determination of trichloroacetic acid-soluble products by reaction with fluorescamine as described by Böhlen and colleagues (29). The extract (typically 10 μg protein) was incubated at 37°C with 100 μg substrate (native or oxidized glutamine synthetase). Increasing the amount of substrate beyond the 1:10 ratio of extract:substrate protein did not change activity. The total volume was routinely 60 μl, allowing two samples to be obtained

[3] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; Mops, 4-morpholinepropanesulfonic acid.

[4] The details of our procedure differ somewhat from that used by Starke-Reed and Oliver (personal communication). They prepared their substrate from hepatocytes harvested from Brown Norway rats ages 20–24 months. An extract was prepared by sonication which was then centrifuged at 18,000*g* for 20–30 min. It was exposed to the iron/ascorbate oxidizing system overnight, after which the preparation appeared cloudy. It was not centrifuged at this point, but cleared during dialysis. The preparation was dialyzed against 5–10 vol of hepatocyte wash buffer (13) for 1–2 h and then stored. Thus, these preparations might contain residual iron and ascorbate which could affect the assays of proteolysis. Proteolysis was assessed at a single time of incubation, typically 1 h, and a blank without test extract was incubated to obtain the background value.

[5] Personal communication, P. Starke-Reed.

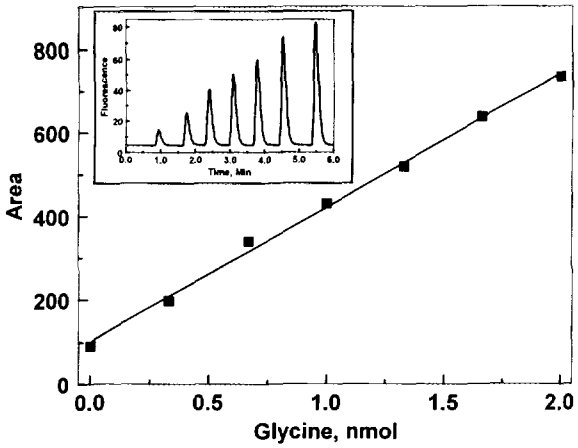

FIG. 1. Calibration curve for the fluorimetric assay. Glycine was derivatized as described under Materials and Methods. The inset shows the tracing from the HPLC fluorimeter, with a peak for each of the 7 standards.

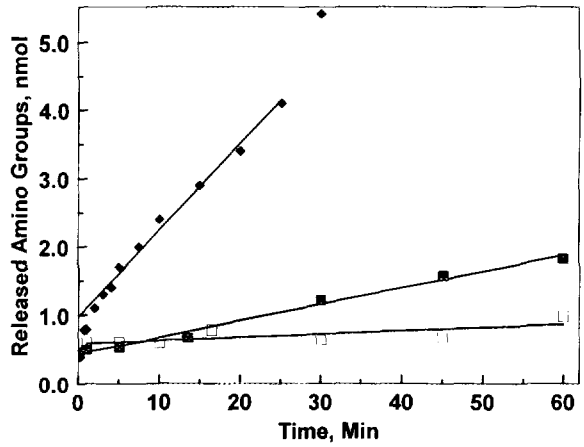

FIG. 2. Time courses for proteolysis of glutamine synthetase. Oxidized glutamine synthetase, 10 μg, with 0.10 μg trypsin (◆). Oxidized glutamine synthetase, 150 μg, with 10 μg liver extract protein (■). Native glutamine synthetase, 150 μg, with 10 μg liver extract protein (□). The regression lines were fit to all points for the liver assays, and for the 2- to 25-min points for the trypsin assay.

at desired times in order to determine the rate of reaction. We usually sampled at 5 and 45 min, with the assay remaining linear for at least 60 min. The assay buffer can be varied as wished. Except where stated otherwise we used 50 mM Hepes, 100 mM KCl, 10 mM $MgCl_2$, pH 7.2. At the desired times, we withdrew 20 μl and added it to a 1.5-ml centrifuge tube containing 80 μl 12.5% (w/v) trichloracetic acid. After mixing by vortex, it was capped and left on ice until all samples were obtained. Then all tubes were centrifuged in a tabletop centrifuge (11,000*g*) for 5 min. Samples were derivatized and analyzed individually from this point on, to assure reproducibility of timing. Eighty microliters of supernatant was withdrawn, taking care not to disturb the pellet. This sample was added to a tube containing 100 μl 2 M potassium borate, pH 10.0, thus assuring that the reaction with fluorescamine occurs at alkaline pH (30). Sixty microliters of fluorescamine was then added while mixing on the vortex. The desired volume (10–100 μl) was then injected into an HPLC detection system. Use of this system instead of a stand-alone fluorimeter allowed reduction of sample volume and thus substrate consumption. It also allowed use of the HPLC integrator to provide a convenient and accurate method for quantitation.

The detector was a Hewlett-Packard Model 1046 HPLC fluorimeter with excitation at 375 nm and emission at 475 nm. No column was used, and the HPLC simply pumped water at 1 ml/min. Data were processed by the attached Hewlett-Packard ChemStation software, but any integrator will work. Samples are injected serially, giving multiple peaks whose areas are integrated (Fig. 1). Determination of the area avoids difficulties associated with measurement of peak heights. The volume of sample injected is thus unimportant and can be adjusted to give an area within the desired range. Glycine, 0–150 ng, was used as a calibration standard, and solutions were treated with trichloroacetic acid in the same way as the extracts. A typical tracing and standard curve are shown in Fig. 1 and typical time courses in Fig. 2. One should note that the method has the potential to remain linear over a wide concentration range because it is a fluorimetric assay.

RESULTS

Activity of glutamine synthetase and glucose-6-phosphate dehydrogenase. The activity of many enzymes has been reported to change during aging, sometimes in a gender-specific fashion (31, 32). In the earlier study of male, Fischer 344 rats,[5] hepatocyte glutamine synthetase, and glucose-6-phosphate dehydrogenase activity decreased by 40–50% between 3 and 26 months of age (13). We confirmed the decrease in activity of glutamine synthetase activity in liver extracts of both male and female Fischer 344 rats (Fig. 3). There was no change in activity of glucose-6-phosphate dehydrogenase in female livers, but a dramatic increase in activity in 26-month old male livers (Fig. 4). This pattern matches that reported by Wang and Mays (33).

Protease activity during aging determined with gluta-

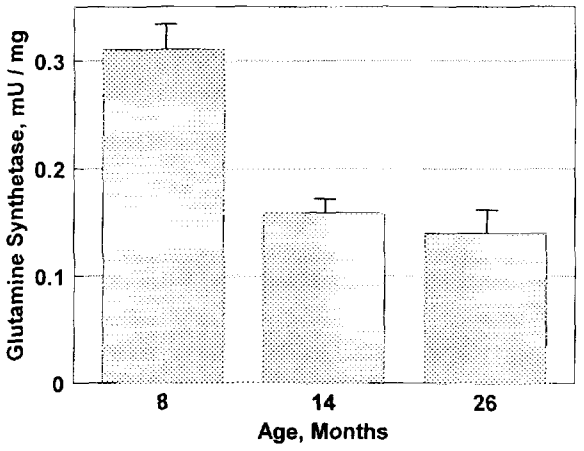

FIG. 3. Glutamine synthetase activity in livers of Fischer 344 rats. Seven animals were assayed at each age. There was no difference between males and females, so the results were combined for this figure.

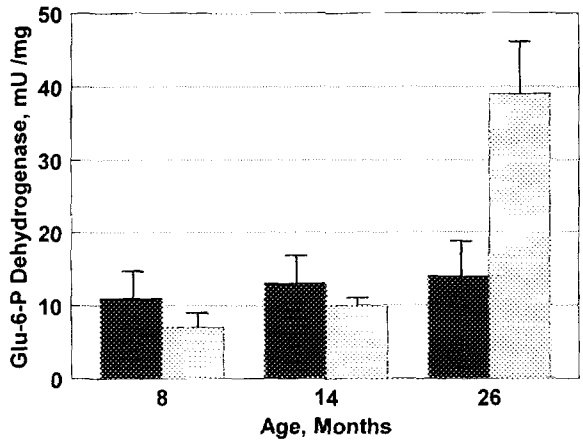

FIG. 4. Glucose-6-phosphate dehydrogenase activity in livers of Fischer 344 rats. Three animals were assayed for each bar shown. Females, dark bars; males, light bars. The activity of 26-month-old males was statistically significantly higher than that of all others.

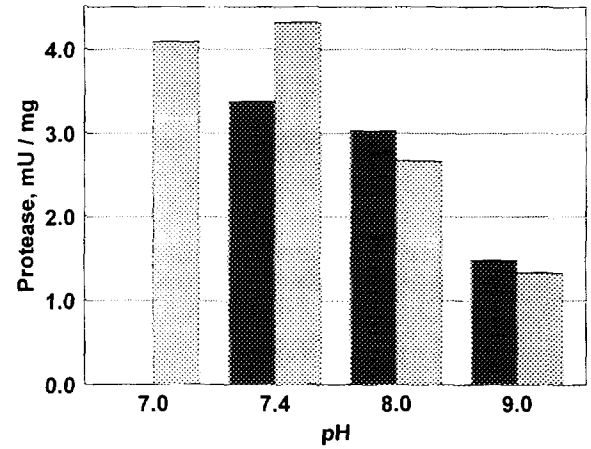

FIG. 6. Protease activity in hepatocytes of Fischer 344 rats. Six months old (dark bars), 26 months old (light bars).

mine synthetase as substrate. Oxidatively modified substrate was degraded at least three times faster than the native substrate (Fig. 5). There was no difference in activities between male and female animals nor during aging. These data were obtained with frozen liver slices from the colony in Japan. Since the results differed from those reported by Starke-Reed and Oliver (13), we also studied homogenates freshly prepared from young and old male rats obtained through the National Institute on Aging. Two strains were tested, Fischer 344/Brown Norway hybrids (6 and 30 months) and Sprague–Dawley (7 and 25 months). Protease activity was maintained during aging, whether or not livers were perfused *in situ* before removal (data not shown). All of the data thus far were obtained with homogenates of whole liver, so we also studied extracts from hepatocytes which were prepared from Fischer 344 male rats. Protease activity from 26 month old animals was as high as that from 6-month-old animals across the tested pH range of 7 to 9 (Fig. 6).

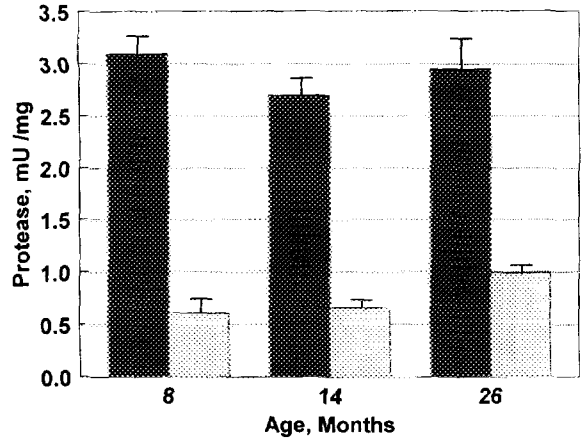

FIG. 5. Protease activity in livers of Fischer 344 rats. Seven animals were assayed at each age. There was no difference between males and females, so the results were combined for this figure. Native glutamine synthetase as substrate (dark bars), oxidized glutamine synthetase as substrate (light bars).

Protease activity determined with oxidized liver protein as substrate. As explained under Materials and Methods, we elected to use a defined substrate in studying the capability of liver extracts to degrade oxidatively modified protein. However, our finding that aged animals maintain proteolytic activity could well be the consequence of the differences between our routine assay and that selected by Starke-Reed and Oliver (13). Their substrate was the soluble extract from hepatocytes of animals over 20 months of age, and oxidized by exposure to metal-catalyzed oxidation.[5] They assayed at pH 2.5, 5.5, 7.4, and 8.8 and an age-related decrease in activity was observed at pH 7.4 and 8.8.

We repeated our studies using oxidatively modified protein prepared from both young and old rat liver (3–6 and 24–26 months).[6] Protease activities were assayed in liver homogenates from rats aged 3 and 26 months, both Sprague–Dawley and Fisher 344 strains. Again, there was no difference in protease activity between the young and old animals (data not shown). This was true whether or not the animals were fasted overnight before killing. The results were the same whether homogenates were prepared by sonication or by the Potter-Elvehjem homogenizer.

The background for this assay was found to be rela-

[6] We could not carry out assays with unoxidized extracts because of their very high endogenous protease activity, confirming the experience of Starke-Reed and Oliver (personal communication).

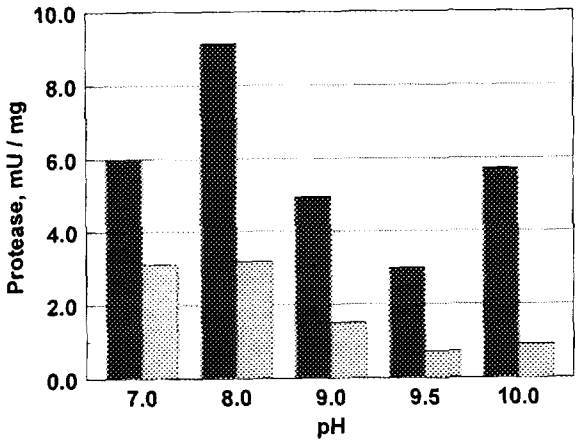

FIG. 7. Endogenous protease activity remaining after 24 h exposure of liver extracts to metal catalyzed oxidation. The rats were Fischer 344/Brown Norway, 7 months old (dark bars), 33 months old (light bars).

tively high as a consequence of residual protease activity in the oxidized substrate. Thus, the activity in the extracts being analyzed contributed at most 30% of the measured fluorescence, consistent with the experience of Starke-Reed and Oliver.[5] Background could be decreased somewhat by gel filtration on a Sephadex-25 column or by preparation of an 80% ammonium sulfate cut followed by dialysis. Interestingly, the background from the oxidized substrate was lower when prepared from old liver than when prepared from young liver (Fig. 7). This observation suggests that protease activity is less resistant to oxidative inactivation in older animals than in younger. Alternatively, exposure to the oxidizing system may generate an inhibitor of the protease, with the old extract generating more than the young. The operation of either mechanism would imply that an increase in oxidative stress during aging could cause a decrease in protease activity (see Discussion.).

Activity of purified multicatalytic protease from young and old animals. The studies of protease activity described above were carried out on crude homogenates. We therefore purified the multicatalytic protease from the liver of young and old rats. Assay of the purified enzymes with oxidized glutamine synthetase revealed no substantive differences in the apparent K_m nor specific activity for enzyme from young and old animals (Fig. 8).

DISCUSSION

The specific activity of some enzymes decreases during aging, although physiologic function need not necessarily decline as a consequence (7, 31). Oxidative modification of proteins is now appreciated to be mediated by a variety of mechanisms, and the general effect on enzymes is loss of catalytic activity (8). The content of oxidatively modified proteins increases during aging, with an exponential increase with age that leads to a particularly dramatic increase in animals approaching their maximum life span (6, 15). Thus, accumulation of oxidatively modified forms may be the basis for the decrease in specific activity of enzymes observed during aging.

Oxidatively modified proteins are generally not repaired but are removed by proteolytic degradation, with available data suggesting that proteolysis is mediated by neutral or alkaline proteases rather than lysosomal acid proteases (18). WI-38 cells maintained in cell culture stop dividing after a certain number of mitotic divisions, a phenomenon referred to as cellular senescence. The activity of acid proteases toward acetylated hemoglobin changed little when the cells reached senescence, but proteolytic activity measured at pH 7.8 became undetectable at senescence (34).

Proteases with specificity for oxidized substrates have been purified and characterized from mammalian tissues as well as bacteria (22, 35). A decline in such protease activity during aging would cause an increase in the steady-state level of oxidized proteins. Recognizing this point, Starke-Reed and Oliver studied the activity of liver extracts from Fischer 344 rats toward oxidatively modified liver protein (13). They demonstrated a decline of activity at pH 7.4 and 8.8 but not at 2.5 or 5.5. Liver from rats age 26 months had less

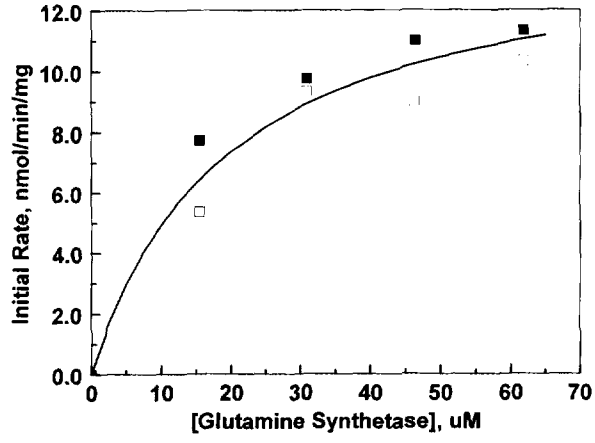

FIG. 8. Substrate–velocity curves for multicatalytic protease purified from male Fischer 344 rats, age 8 months (■) or 24 months (□). The assays were performed with 10 μg protease and the indicated amount of oxidized glutamine synthetase as substrate. Aliquots were taken at 5, 15, 30, and 45 min for determination of the initial rate by linear regression with a least-squares fit. The correlation coefficients were always >0.99, confirming that the rate of proteolysis was constant during the assay. The incubation solution was 50 μl of 50 mM Hepes, 100 mM KCl, and 0.8 mM $MgCl_2$. Ten-microliter aliquots were withdrawn, mixed with 40 μl of 12.5% trichloroacetic acid, and assayed as described under Materials and Methods.

than 30% of the activity found at 3 months. Recently, Agarwal and Sohal (17) confirmed these results in Sprague–Dawley rats, using the same assay except that irradiated bovine albumin was the substrate. Interestingly, they found no age-dependent change in proteolytic activity in extracts from rat heart, rat brain, nor whole fly.

We planned to explore the mechanism of that decrease and considered that it might have resulted from (i) a decrease in the amount of protease, with the remaining enzyme having normal specific activity; (ii) accumulation of a protease with decreased average specific activity, perhaps as a consequence of oxidative modification; (iii) accumulation of an inhibitor of the protease.

We thought it useful to develop an assay with a purified protein substrate which would allow comparison of activity on the native and oxidatively modified forms of the protein. This strategy was utilized previously in the detection and purification of proteases with high specificity for the oxidized form (22, 35). The approach was developed initially during studies of proteolysis in *Escherichia coli,* and the protein substrate was glutamine synthetase from *E. coli* which had been radiolabeled biosynthetically (22). Proteolysis was measured by following the release of trichloroacetic acid-soluble radioactivity from the native or oxidized proteins. Rivett adopted the same assay for the purification and characterization of rat liver proteases specific for oxidized proteins, notably the multicatalytic protease (18). The multicatalytic protease degraded only the oxidized form of glutamine synthetase, despite its bacterial origin. The capability of distinguishing between native and oxidized mammalian proteins was also demonstrated. A similar discrimination for bacterial glutamine synthetase was demonstrated *in vivo* with cultured hepatoma cells (36). Microinjection of native and oxidatively modified *E. coli* glutamine synthetase revealed that the native form had a half-life of about 60 h while the oxidatively modified form was degraded 10 times faster. Glutamine synthetase can be produced in gram quantities, is easily purified, and can be reproducibly modified by metal-catalyzed oxidation (19). It was therefore selected as the substrate for these studies. Use of an HPLC fluorimetric detector provided sufficient sensitivity to eliminate the need for a radiolabeled substrate. Two timed samples can be obtained from an incubation mixture containing 10 μg sample protein.

Using this assay, we demonstrated that older rats maintain liver protease activity. The same result was obtained with Fischer 344 rats from colonies in Japan and the United States, as well as Sprague–Dawley rats from the United States. It seemed possible that failure to detect a decrease in activity was due to the use of oxidized glutamine synthetase as substrate, in place of oxidized bulk liver protein. We therefore returned to the original assay with oxidized liver protein as substrate, and again documented maintenance of protease activity during aging. We also purified the multicatalytic protease from young and old animals and found no significant differences in the specific activity or K_m for oxidized glutamine synthetase.

A reasonable explanation for the difference between our results and those reported earlier is that the animals studied by Starke-Reed and Oliver (13) or by Agarwal and Sohal (17) are different from those available to us now. Subtle changes in animal care procedures, in food composition, or in exposure to xenobiotics could have caused the change. Changes in the biology of animal colonies are well known, and such changes are presumably more likely to be observed in animals held for the longer times required in studies on aging (37).

We conclude that the intrinsic catalytic activity of rat liver neutral or alkaline proteinase(s) does not decline in the older animals currently available for study. The results presented here and by others (17) emphasize that the proposal that aging is accompanied by a decline in proteolytic capacity requires additional experimental verification. Elucidation of differences among studies and within tissues would certainly enhance our understanding of the biology of aging.

ACKNOWLEDGMENT

We thank Dr. Pamela Starke-Reed for many helpful discussions and for her support in pursuing these studies.

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