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Age-Dependent Changes in Proteins of *Drosophila melanogaster*

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Several molecular theories of aging postulate that there are age-dependent changes in gene expression and that these changes contribute to the reduction in the viability of senescent cells. High-resolution, semiautomated, quantitative two-dimensional gel electrophoresis of many soluble proteins was used to test this hypothesis in *Drosophila*. Two-dimensional protein gel patterns were analyzed for each of three age groups of [^{35}S]methionine-labeled adult male *Drosophila melanogaster*, which, except for their spermatocytes, consist entirely of fixed postmitotic cells. Seven relatively abundant polypeptides expressed in middle-aged (28-day-old) flies were absent in both young (10-day-old) and old (44-day-old) flies. Quantitative analyses of an additional 100 polypeptides were carried out by computer-assisted microdensitometry of fluorograms of the gel preparations. These analyses revealed a significant age-related heterogeneity in the quantitative distribution of radiolabel in these proteins. The data indicate that the qualitative pattern of gene expression is identical in young and old flies, but that profound quantitative changes occur in the expression of proteins during the *Drosophila* life-span.

ONE OF THE UNANSWERED QUESTIONS in gerontological research is whether significant alterations in gene expression occur with age. Thus, several current molecular theories of aging postulate that age-dependent changes occur in proteins and that such changes contribute to the reduction in the viability of senescent cells (*1–5*). Many aspects of gene expression have been evaluated in an attempt to clarify this issue (*6*). So far, studies aimed at detecting translational errors as a function of age have failed to provide any convincing evidence in favor of this hypothesis (*7, 8*). Observations in support of mutational theories of aging are also lacking (*9–11*). Neither amino acid sequence nor amino acid compositional alterations have been found to be age-dependent (*12*). In addition, previous studies involving the use of two-dimensional (2-D) gel electrophoresis to search for protein changes with senescence have generally been restricted to qualitative assessments, with the exception of several attempts at quantitative analyses of only a few polypeptides (*13, 14*).

Although the methodology used in these studies can provide significant information on variations in gene expression, such techniques are of limited value because alterations in the expression of proteins in old animals may be quantitative rather than qualitative. So far, no rigorous quantitative evaluation of polypeptides from animals of different ages, separated by high-resolution 2-D gel electrophoresis, has been reported. This is due in part to the inability of many laboratories to perform these types of analyses. In contrast to classical studies of protein turnover, semiautomated, quantitative 2-D gel electrophoresis provides an accurate measure of the magnitude of expression of a large array of individual polypeptides.

We used the imago of *Drosophila melanogaster* as a model of aging to look for qualitative changes in the expression of proteins by high-resolution 2-D gel electrophoresis (*15–17*). In spite of the sensitivity of this technique and the many proteins examined, the fluorograms did not provide evidence that there are charge differences in any single protein between young and old flies. Moreover, the analyses did not reveal changes in the molecular weight of any of the proteins from any of the age groups, that is, the qualitative pattern of gene expression by young and old *Drosophila* was identical. Therefore our data do not support theories that invoke molecular weight changes (protein cross-linking) or charge alterations (error catastrophe, mutations, or deamidation) as the mechanism for senescence (*2–5*).

Although one low molecular weight pro-

Table 1. Quantitative comparisons of all gels. Ten gels from each age group were analyzed. Two exposures of each gel were made to quantify both faint and dark spots without saturating the film. Standard deviations, calculated from the data in Fig. 2, were obtained from the protein spot mean for each protein within its group of gels. In a given comparison, the standard deviation is calculated from the distribution of differences of the means between the two groups over the 100 spots. The protein quantities are normalized, so each quantity is the percentage of the total protein quantity on the gel, which makes the mean of these differences 0. The greater than twofold differences indicate the number of proteins with at least twice as much or half the label of the corresponding protein in the group compared. Qualitative differences represent the number of proteins in which the intensity is at least 20 times greater than its counterpart in the comparative sample. The table also indicates the reproducibility of the method, as determined from data obtained as described in the legend to Fig. 2A.

Compared	Quantitative data				Qualitative data	
	Proteins compared	Correlation coefficient	Standard deviation ($\times 10^{-3}$)	Proteins more than twofold different	Proteins compared	Differences
Young versus young	100	0.97	1.45	0	>500	0
Middle versus middle	100	0.97	1.65	0	>500	0
Old versus old	100	0.98	1.55	0	>500	0
Young versus middle	100	0.87	3.55	2	>500	7
Young versus old	100	0.77	5.60	10	>500	0

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tein migrated to a more acidic position in middle-aged flies, its mobility was identical in both young and old flies (Fig. 1). Also, seven relatively abundant proteins appeared in middle-aged flies that were not detectable in the gel preparations from young or old *Drosophila* (Fig. 1). However, overexposure of the fluorograms from the latter age groups revealed that these polypeptides were expressed, but at greatly reduced rates. According to Garrels (*18*), quantitative differences of such a magnitude represent qualitative changes, but in this instance they are not a sign of senescence. Most likely, their appearance reflects the maturation of enzymes involved with flight activity. It has been shown, for example, that certain enzymes, such as cytochrome oxidase, reach peak activity during middle age in certain insects (*19*). The dramatic increase in the expression of these proteins can be viewed as a programmed event in the life-span of *Drosophila*. Thus, our data do not show an age-dependent loss of individual proteins or the appearance of new ones, a finding invalidating stochastic molecular aging theories that predict that qualitative differences should increase with age. Moreover, program theories do not provide an adequate explanation of these data showing that senescence in *Drosophila* is not the result of the expression of specific aging proteins.

To test for more subtle quantitative differ-

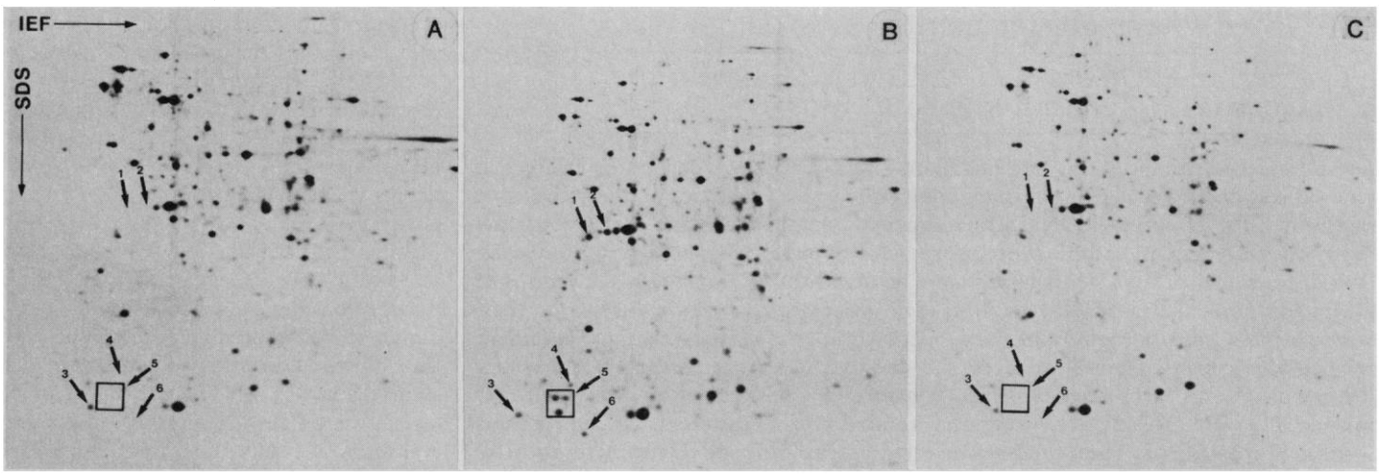

Fig. 1. Representative 2-D fluorograms of whole-cell proteins from adult male *D. melanogaster* (Ore R Strain) raised at 25°C. Proteins are from young (10-day-old) (A), middle-aged (28-day-old) (B), and old (44-day-old) (C) flies at the beginning of the labeling period. The mean life-span for this population was 32.5 days. To minimize sample variation, the three age groups were derived from the same parental population of a highly inbred strain of *Drosophila*. Each group was labeled continuously for 5 days with [^{35}S]methionine when it reached the appropriate age and then immediately frozen at –76°C (*24*). After all three age groups of flies were labeled, samples were subjected to electrophoresis in a random order so as to minimize gel-to-gel and run-to-run variation. The 2-D gel electrophoretic technique was performed as described by O'Farrell (*25*) with the Anderson Iso Dalt system (*26*). Each gel was loaded with 2×10^6 dis/min to minimize the quantitative variation in the lower intensity spots. Ten gels were prepared for each age group from randomized samples. Fluorograms were prepared (*27*) and dried with the aid of a Hoeffer SE1150 slab gel dryer and exposed to Kodak XAR-2 x-ray film. The films were developed with a Kodak M5A-N X-Omat processor. With each of the ten gel preparations for each age group, two exposures were made so that spots of lower intensity could be quantified. The data from the two exposures of each gel were combined for analysis because calibration strips were used to convert integrated film density to radioactive counts (*18*). Arrows indicate the proteins whose expression or modification was confined to the middle age group.

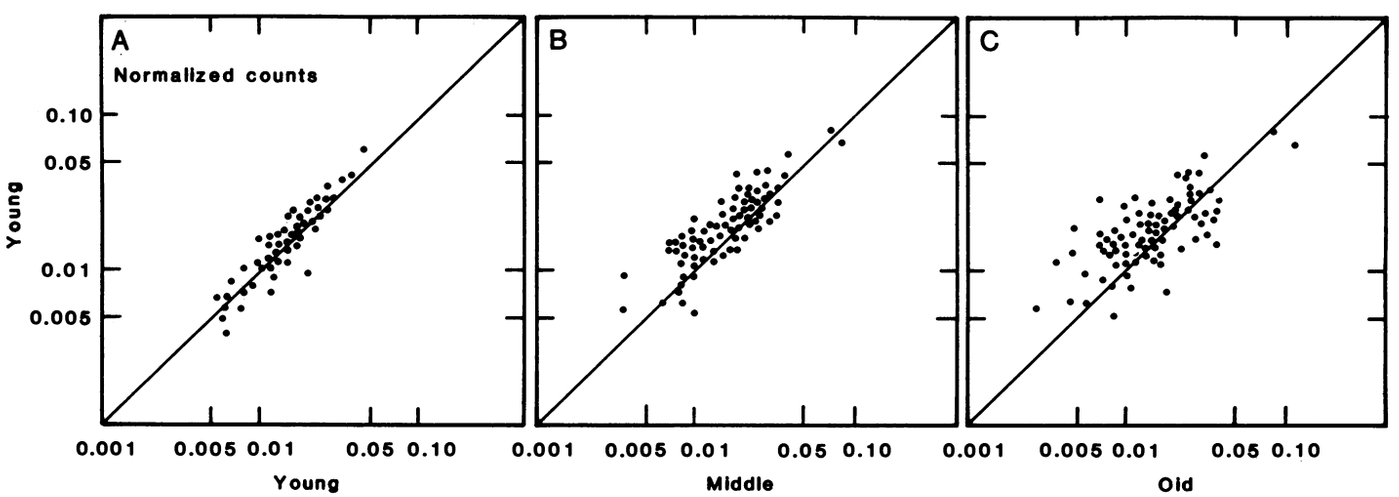

Fig. 2. Scatter plots showing the increase in the heterogeneity of labeling of individual polypeptides with advancing age. The microdensitometric data have been converted to normalized radioactive counts and the mean values across the gels were calculated for each of the 100 proteins. In (A) the ten gels for the young group were randomly divided into two groups of five gels each; individual protein spots were averaged for each group, with the mean of each protein from one group plotted on the *x*-axis and the mean of the same protein from the other group on the *y*-axis. In (B) the mean value for each protein from all ten gels in the middle-aged group is plotted on the *x*-axis and the mean for the same proteins from the ten gels in the young group is plotted on the *y*-axis. In (C) the data for the old flies was also compared with those for the young flies, as described for (B).

ences in the label content of individual polypeptides of the three age groups, we analyzed 100 of the most abundant proteins in ten gel preparations of each group by computer-assisted microdensitometry (*18, 20*). The quantitative analyses were carried out as previously described, with the final calculations based on the normalized integrated density of the protein spots on the fluorograms (*18, 20*). The density of each spot was expressed as a fraction of the total for all the spots on a gel. To determine reproducibility, we compared ten fluorograms for the same age group by dividing them into two groups of five gels each. Individual protein spots were averaged for each of the two groups and then compared. Figure 2A and Table 1 show the increase in heterogeneity with age. After this level of reproducibility was obtained, the same procedure was used for quantitative comparisons of the three age groups, except that the data from all ten gels of each group were used (Fig. 2 and Table 1). These data show a significant increase in the heterogeneity of expression of these proteins as a function of age.

The mechanism underlying quantitative deterioration of protein expression with advancing age is not known, but it could stem from any of a number of postulated molecular events, including changes in DNA binding proteins, DNA superstructure, or conformational alterations in chromatin (*21, 22*). Each of these events could lead to quantitative shifts in gene expression. Different turnover rates in old *Drosophila* could also result from an accumulation of specific inhibitors of protein synthesis. Age-related activation or repression of gene expression by such inhibitors has been suggested, and is consistent with our findings (*22, 23*). An alteration in the turnover or synthesis rate of one or more critical proteins that regulate the expression of several other proteins might lead to heterogeneity in the production rates of all the polypeptides in a given feedback loop. With time, control would gradually degenerate until cell homeostasis could no longer be maintained. In this case, senescence would result from the gradual loss of the normal quantitative pattern of gene expression due to changes in the synthesis rates of a few key proteins.

Alternatively, since the *Drosophila* imago consists of different organs—though all are made of fixed postmitotic cells—the aging rate of these organs (individual cell types or even subcellular organelles of each cell type) may differ. Such differences in the aging rate could be expected to result in differences between young and old flies in the isotope content of the same proteins.

It is not yet possible to assign to our findings a primary or secondary role in the aging process. They do appear to represent the first evidence of age-induced quantitative heterogeneity at the molecular level. However, a significant deviation from the normal concentrations of body constituents typically has detrimental effects on the function of cells, organs, or an entire organism—whether such alterations are caused by mutations or by damage to individual animals (*10*).

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