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Aging results in an unusual expression of *Drosophila* heat shock proteins

(canavanine/two-dimensional polyacrylamide gel electrophoresis)

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ABSTRACT **We used high-resolution two-dimensional polyacrylamide gel electrophoresis to evaluate the effect of aging on the heat shock response in *Drosophila melanogaster*. Although the aging process is not well understood at the molecular level, recent observations suggest that quantitative changes in gene expression occur as these fruit flies approach senescence. Such genetic alterations are in accord with our present data, which clearly show marked differences in the synthesis of heat shock proteins between young and old fruit flies. In 10-day-old flies, a heat shock of 20 min results in the expression of 14 new proteins as detectable by two-dimensional electrophoresis of [^{35}S]methionine-labeled polypeptides, whereas identical treatment of 45-day-old flies leads to the expression of at least 50 new or highly up-regulated proteins. In addition, there is also a concomitant increase in the rate of synthesis of a number of the normal proteins in the older animals. Microdensitometric determinations of the low molecular weight heat shock polypeptides on autoradiographs of five age groups revealed that their maximum expression occurs at 47 days for a population of flies with a mean life span of 33.7 days. Moreover, a heat shock effect similar to that observed in senescent flies occurs in young flies fed canavanine, an arginine analogue, before heat shock.**

Many of the modern theories of molecular aging imply, directly or indirectly, that age-dependent changes occur in the molecular configuration or expression of proteins and that these changes contribute to the diminution in the viability of senescent cells (1). In essence, these theories can be divided into three broad categories: (*i*) those that predict posttranslational modifications such as protein crosslinkage (2) and deamidation or glycosylation (3); (*ii*) those that predict alterations in primary structure, such as the mutational theories (4–7) and the error-catastrophe hypothesis (8); and (*iii*) those that predict specific changes in the pattern or timing of protein expression, such as the genetic-program theories (9). Several investigations have demonstrated that posttranslational changes do indeed occur during senescence (10–12); however, unequivocal evidence showing an age-dependent effect on the fidelity of protein synthesis has not been reported (1, 13). Previous studies in this laboratory gave no evidence that aged *Drosophila* express altered polypeptides (1). Two-dimensional gel electrophoresis data indicated that the qualitative pattern of protein expression was identical in young and old flies, but there were significant quantitative changes in the expression of proteins with age in the unstressed imago of *Drosophila melanogaster*.

The expression of "new" polypeptides was also observed in non-heat-shocked senescent flies after exposure to inhibitors of mitochondrial function such as nonactin (14). These results suggest that qualitative changes in gene expression can be induced in aged animals by a metabolic or chemical stress. Thus, general perturbations of homeostasis, such as those induced by heat shock, should provide insight into mechanisms that lead to alterations in gene expression during aging. Heat shock of most eukaryotic cells, including those of *Drosophila*, induces a small set of genes that code for the appropriately named "heat shock proteins" (15). These proteins can be accurately analyzed by electrophoresis in polyacrylamide gels. Therefore, we decided to investigate whether a brief heat shock to young and aged fruit flies results in different polypeptide patterns upon two-dimensional polyacrylamide gel electrophoresis. The fruit fly *D. melanogaster* is an ideal model for such studies on senescence because (*i*) it has a relatively short life span, (*ii*) large colonies can be economically maintained under controlled laboratory conditions, (*iii*) the somatic cells of the adult are composed of postmitotic cells, (*iv*) the species is available in highly inbred and outbred strains as well as numerous well-characterized mutants, and (*v*) the imago appears to show many if not all of the manifestations of cellular senescence observed in mammals (16).

With the exception of the work by Hiromi and Hotta (17), the literature contains very little information on polypeptide expression in response to such stresses of *Drosophila* imagos. Moreover, there have been no reports on this response in any aging animal model. Our data show that a brief heat shock applied to aged flies results in an unexpected expression of unusual polypeptides detectable by two-dimensional gel electrophoresis.

MATERIALS AND METHODS

***Drosophila* Cultures.** Male *D. melanogaster* of the Oregon-R strain, which were obtained from the National Aeronautics and Space Administration Ames Research Center in 1981, were maintained at 24° ± 1°C in half-pint bottles containing Instant Drosophila medium (Carolina Biological, Burlington, NC). Mean survival time was 33.7 days; percentiles are 0.50, 34 days; 0.75, 43 days; 0.90, 49 days. n = 225 flies.

Heat Shock. Heat shock was applied by transferring groups of flies of the various age groups in thin-walled 35-ml glass vials from their usual 24°C environment into a 37°C dry-air incubator for 20 min; under these conditions, the temperature of the air inside the vial rises gradually and reaches 35°C after 15 min (Fig. 1). In addition, several groups of flies were fed canavanine before and during heat shock as follows: approximately 20 flies were allowed to feed from a piece of filter paper impregnated with a 25% sucrose solution containing canavanine at 200 mg/ml for 2 hr before heat shock and during the 20 min of heat shock. After the heat shock, all groups were immediately exposed to filter paper saturated

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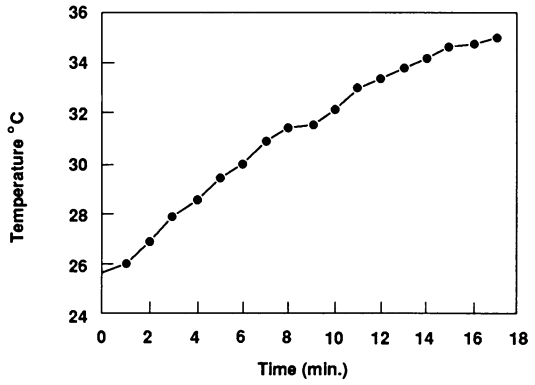

FIG. 1. Temperature increase inside the 35-ml glass vials in which adult male *D. melanogaster* were heat-shocked. Flies were placed inside thin-walled 35-ml glass vials at 24°C and the vials containing the flies were transferred into a 37°C dry-air incubator for 20 min.

with [^{35}S]methionine (specific activity, 1103 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) for 2 hr at 24°C, and the protein samples were prepared for gel electrophoresis as described (18, 19). Pilot studies in our laboratory have shown that this schedule is best-suited for the visualization of the heat shock proteins from *Drosophila* imagos in two-dimensional gels. Non-heat-shocked groups were maintained in the 35-ml vials at 24°C for the 20 min prior to radiolabeling.

Two-Dimensional Gel Electrophoresis. The technique was essentially that of O'Farrell (20) and made use of the Anderson Iso-Dalt system (Electro-Nucleonics, Oak Ridge, TN) (21). After the labeling period, flies were homogenized in O'Farrell's lysis buffer A in a Dounce glass homogenizer. After the first-dimension (isoelectric-focusing) gels were prefocused for 1 hr at a constant voltage (550 V), 10-μl samples ($\approx 10^6$ dpm) were applied under the buffer at the negative ends of the gels and then run at 550 V for 20 hr. The voltage was then increased to 1000 V for an additional hour. Immediately after this, the gels were removed from the glass tubes and incubated for 1 hr in Anderson's equilibration buffer (21). Gels were then either run immediately in the second dimension or frozen in this buffer at −76°C.

Unless otherwise indicated, all second-dimension electrophoretic procedures were based on O'Farrell's original technique (20) and were performed with the aid of Anderson's Iso-Dalt system (21). The second-dimension gels contained 10% acrylamide and 1% $NaDodSO_4$. After electrophoresis, gels containing radiolabeled proteins were dried with the aid of a Hoeffer model SE1150 slab-gel dryer. The dried gels were exposed to Kodak XAR-2 x-ray film for various times up to 2 weeks. The resulting autoradiographs were developed with the aid of a Kodak model M5A-N X-Omat automatic film processor.

For quantitation of designated spots, specific areas of the autoradiographs were digitized with an Optronics P-1000 film scanner at a pixel size of 200 μm. The abundance of the polypeptides quantified was measured by integrating the observed counts over the pixels in an area surrounding each spot on the gel. Such areas are determined by an investigator using an interactive graphics system. This procedure has been described in detail (22).

RESULTS

Fig. 2*A* is an autoradiograph of [^{35}S]methionine-labeled polypeptides from 10-day-old flies maintained at 25°C. Fig. 2*C* is an autoradiograph of 45-day-old flies labeled and maintained under the same physiological conditions. Despite the high resolution of the two-dimensional electrophoretic technique, neither charge nor molecular weight differences could be found to exist between any of the polypeptides from young and old flies—i.e., the qualitative pattern of gene expression persists into old age. This is consistent with previous results with flies (1, 18), nematodes (23), and rat brain tissue (24) showing that the two-dimensional gel protein patterns of young and old animals are the same.

However, these findings are in sharp contrast to the differences that appear when the flies of the two age groups are subjected to a brief heat shock. The autoradiograph of proteins from young heat-shocked flies indicates that the stress of exposure to 20 min in a 37°C incubator results in the synthesis of 14 new proteins (Fig. 2*B*). In comparison with young heat-shocked flies, an identical heat shock has a much more profound effect on old flies. Here, this stress results in the appearance of at least 50 new or up-regulated radiolabeled proteins on the autoradiograph (Fig. 2*D*). Also, most of the heat shock proteins that are common to both young and old flies are synthesized at higher rates in old flies. Furthermore, in contrast to a typical heat shock response where a brief heat shock results in the diminution of the non-heat-shock proteins (15), heat-shocked senescent flies contain more radioactivity in their non-heat-shock proteins than do either the non-heat-shocked senescent flies or the heat-shocked young flies.

Fig. 3 depicts the gel pattern of the major low molecular weight heat shock proteins of flies from five different age groups. The proteins in boxes 3 and 4 of Fig. 4*A* are of $M_r \approx$ 28,000 and the isoelectric points of all the low molecular weight heat shock proteins range from about 5 to 7.5 (left to right, respectively). The gel patterns clearly indicate that the most abundant synthesis occurs in 47-day-old flies and that it then declines; protein synthesis by 54-day-old insects (those that have reached 95% of their maximal life span) is lower than that observed with 26-day-old insects. This is consistent with our findings that protein synthesis by flies at such an advanced age is greatly reduced, even in the absence of any stress (unpublished data). The microdensitometric data for the selected low molecular weight spots of the gels in Fig. 3 are displayed in Fig. 4*B*. The synthesis of several minor heat shock proteins is increased by a factor of about 5 in 47-day-old flies when compared to their counterparts in young flies. The data in Fig. 4 show that the synthesis of heat shock proteins is greatest in 47-day-old flies. In 54-day-old flies the levels are similar to those seen in younger flies.

Fig. 5 illustrates the effect of heat shock on young flies previously fed with the arginine analogue canavanine. It is particularly striking that many of the proteins synthesized by old but not by young heat-shocked flies now also appear in the young flies fed canavanine prior to heat stress. Moreover, there is a concomitant overall increase in the relative rates of synthesis of the heat shock proteins normally seen in this age group. These results suggest that the gel pattern observed with young flies that have been fed canavanine can mimic to some extent that produced by old heat-shocked flies. However, it should be pointed out that the two-dimensional gels of young flies administered canavanine prior to heat shock are similar, but not identical, to the protein pattern of aged heat-stressed flies. This similarity extends primarily to the family of low molecular weight heat shock proteins.

DISCUSSION

The mechanism responsible for the observed profound differences in the heat shock response between young and old insects is unknown; however, our data provide strong evidence that significant alterations in gene expression are brought about in aged *Drosophila* in response to an environ-

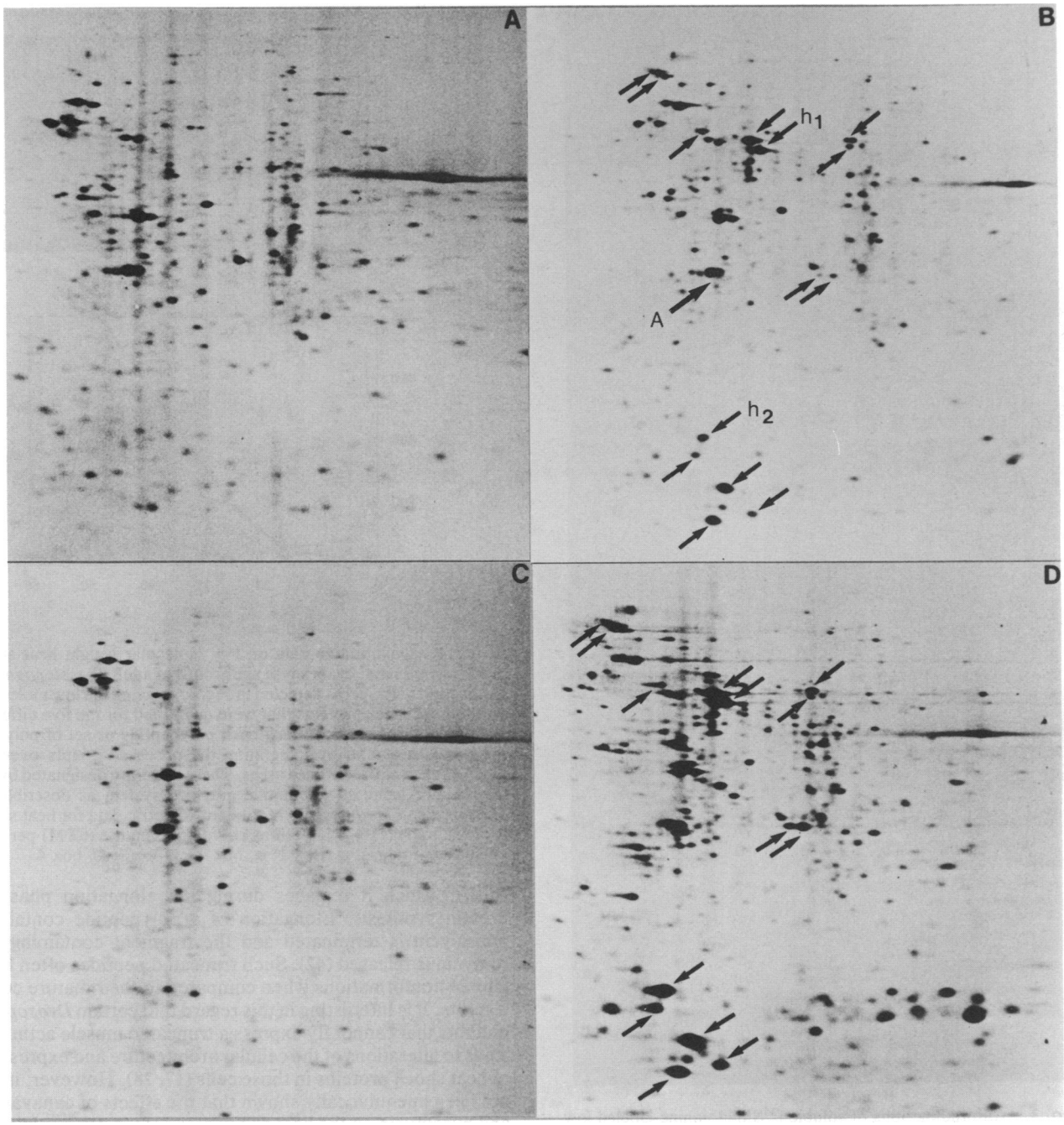

FIG. 2. Autoradiographs of two-dimensional patterns of soluble [^{35}S]methionine-labeled polypeptides from adult *D. melanogaster*. (*A*) Ten-day-old flies. (*B*) Ten-day-old flies after heat shock. (*C*) Forty-five-day-old flies. (*D*) Forty-five-day-old flies after heat shock. Arrows indicate the heat shock polypeptides that are expressed in both age groups. Arrow A, actin (M_r 41,000; pI 5.3); arrows h_1 and h_2, heat shock polypeptides of M_r 70,000 and 28,000, respectively.

mental stimulus. The unexpected appearance of an unusually large number of heat shock proteins in old flies may be promoted by an increase in the number and concentration of heat-denaturable proteins in senescent cells; such polypeptides (those with abnormal conformations) are thought to be important in the induction of the heat shock response (26–30). This notion is supported by the finding that abnormal proteins injected into frog oocytes can indeed serve as triggers of the heat shock response (30). Moreover, the assumption that the presence of abnormal proteins is important in the observed unusual heat shock response in aged organisms is also supported by gerontological data that suggest an increase in the number of such proteins with age (11, 31–34). Several proteins isolated from tissues of old animals show altered conformational and heat-denaturation characteristics. Also, Gracy *et al.* (11) provided convincing evidence for the appearance of altered enzymes during aging. These investigators showed that deamidation of asparagine-71 and asparagine-15 of triose-phosphate isomerase occurs with age and that this leads to a destabilization of the subunit interactions, so that spontaneous dissociation of the enzyme is favored. If altered protein molecules are indeed responsible for the observed unusual age-related heat shock response, it should be possible to provoke a similar effect in young flies by increasing the intracellular level of altered polypeptides. To test this idea, we introduced an amino acid analogue of arginine, canavanine, into the proteins of young flies before heat shock; canavanine is known to be incorporated into

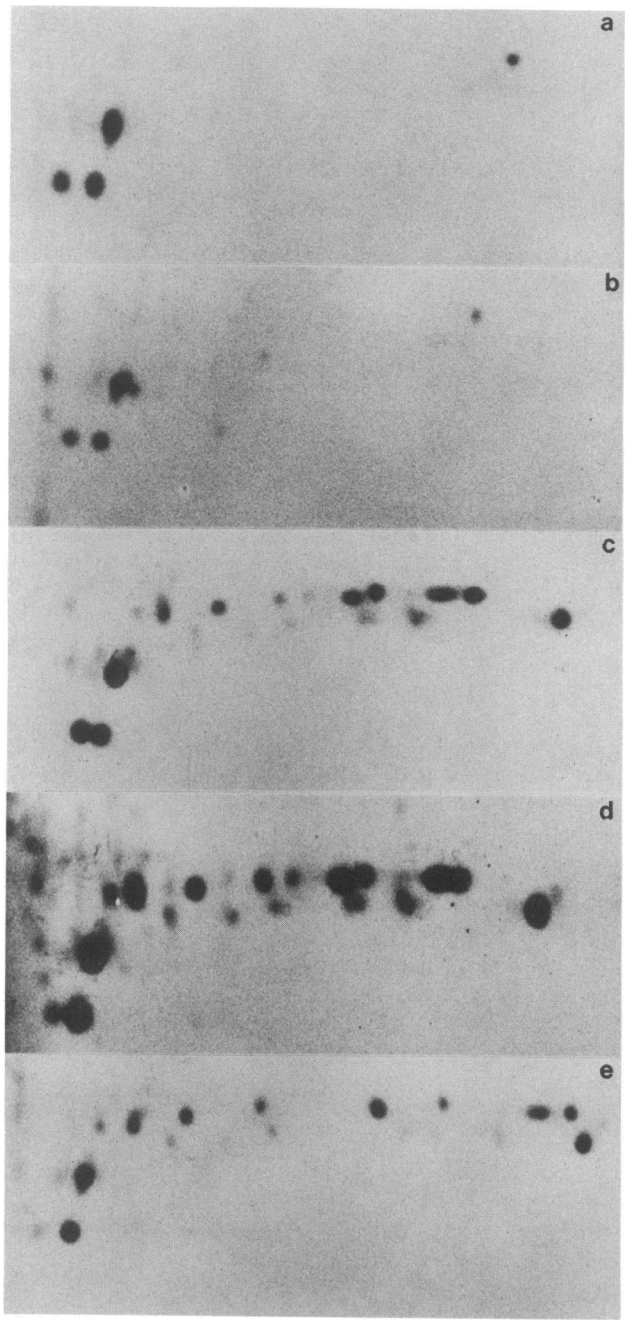

FIG. 3. Autoradiographs of soluble [^{35}S]methionine-labeled low molecular weight heat shock proteins from adult *D. melanogaster* of various ages. Flies were 5 (*a*), 16 (*b*), 26 (*c*), 47 (*d*), or 54 (*e*) days old. Unlike the gels depicted in Figs. 2 and 5, these gels were prepared according to the recipe of Garrels (25).

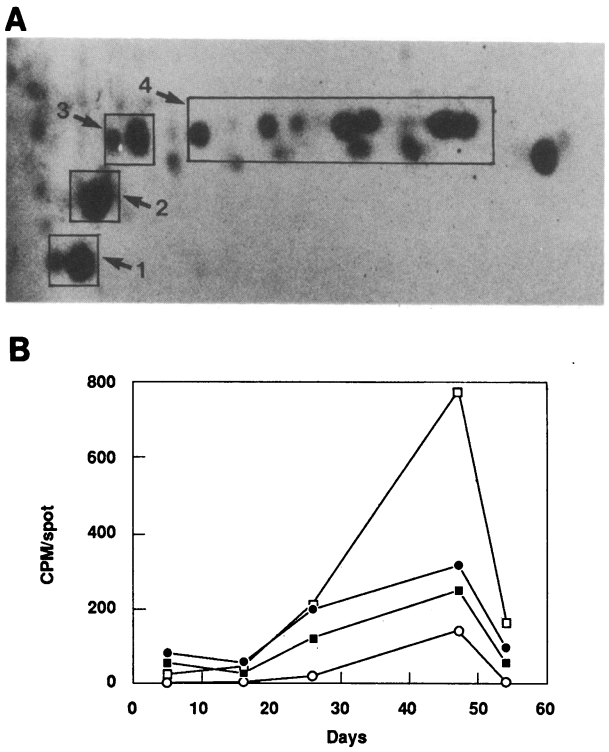

FIG. 4. Quantitative data on low molecular weight heat shock proteins expressed by different age groups of adult *D. melanogaster*. (*A*) Section of the gel as depicted in Fig. 3, showing the low molecular weight heat shock proteins that were quantified for the five different age groups. The abundance of each polypeptide or set of polypeptides was measured by integrating the observed counts over the pixels in each of the outlined areas. These areas are designated by the investigator using an interactive graphics system as described in *Materials and Methods*. (*B*) Microdensitometric data for heat shock proteins in boxes 1–4 plotted as counts per minute (CPM) per spot versus age in days. ■, Box 1; ●, box 2; ○, box 3; □, box 4.

proteins of bacteria and animal cells (35–38), where it leads to abnormal conformations of the peptides (39–41). We have found that canavanine is used for peptide synthesis in *Drosophila* (unpublished observation). This observation is supported by data presented in Fig. 5*A*, which show a number of proteins with altered isoelectric points in the autoradiographs from flies fed canavanine. However, in addition, Fig. 5 also shows the unusual heat shock proteins expressed in canavanine-fed young flies that were subsequently submitted to heat shock. It is evident that these young flies now express a number of heat shock proteins typically found only in older flies after exposure to elevated temperatures. Moreover, flies fed puromycin exhibit a similar response—i.e., enhanced synthesis of heat shock proteins (data not shown). Puromycin is a structural analogue of the 3′-terminal end of aminoacyl-tRNA, which it replaces during the elongation phase of protein synthesis. Elongation of a polypeptide containing puromycin is terminated and the fragment containing puromycin is released (42). Such truncated peptides often have altered conformations when compared to their mature counterparts. It is interesting in this regard that certain *Drosophila* mutants that cannot fly express a truncated muscle actin that leads to alterations of the cellular architecture and expression of heat shock proteins in those cells (17, 28). However, it has not been unequivocally shown that the effects of canavanine and puromycin on the heat shock response in *Drosophila* are mediated by proteins with altered conformation. Nevertheless, these results suggest that the increase in the amount of known heat shock proteins and the unexpected appearance of additional heat shock proteins in senescent animals may, at least in part, be causally related to an accumulation of conformationally altered peptides in aging, postmitotic cells.

To our knowledge, the unusual heat shock response that we have observed in aged flies has not been reported previously. In fact, Tsuji *et al.* (43) noted very little difference in heat shock protein pattern between "young" and "old" fibroblasts *in vitro*. Unfortunately, their cell model is not analogous to ours and their findings are thus not comparable to our observations with adult fruit flies. The somatic tissue of *Drosophila* imagos is composed in its entirety of nondividing cells, whereas old fibroblasts are still capable of mitosis. As is well recognized, senescence of multicellular organisms is brought about by the deterioration of the function of those cell types that do not adequately renew themselves (44).

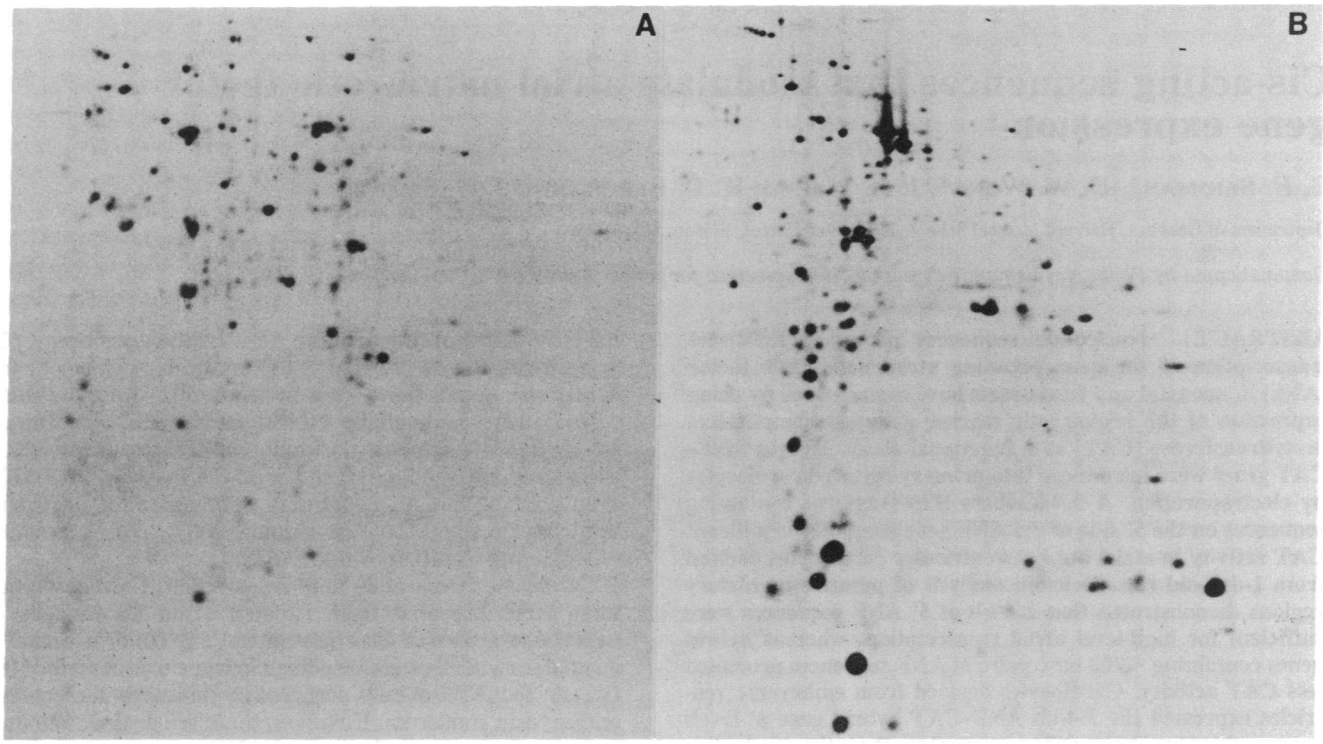

FIG. 5. Autoradiographs of soluble labeled proteins from young flies fed canavanine. (*A*) Ten-day-old flies were fed canavanine (200 μg/ml in 20% sucrose) for 2 hr and then fed [^{35}S]methionine for 2 hr. (*B*) Same treatment as in *A* except that flies were exposed to heat shock (placed in a 37°C incubator for the last 20 min of the canavanine exposure) before radiolabeling.

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