Developmental Regulation in Drosophila melanogaster

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Abstract

An examination by time lapse movie of the process of pupation in Drosophila melanogaster shows a series of muscular contractions to effect breakage of the tracheae and subsequent head invagination.

In order to gain an understanding of the biochemical changes during pre-pupal development, salivary glands from animals in the late larval to the pupal stages were pulse-labeled with methionine or cysteine and the patterns of the proteins were analyzed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Many of the proteins detectable by Coomassie are generally present in all stages from late larvae to pupae. However, autoradiographs of the same gel show rapid rates of synthesis in the prepupal stages especially for a set of low molecular weight proteins (3K-12K). These small polypeptides show a dramatic reduction in their rates of synthesis at the time of pupation.

Analyses of proteins from the salivary glands of animals injected with labeled methionine and cysteine show a general maintenance of most, if not all, of the proteins except for the low molecular weight components which diminish drastically at pupation. Results also suggest a transport of these small polypeptides from the salivary glands to the fluid between the pupal case and the prepupal cuticle (pupation fluid). These low molecular weight proteins are very basic and each component actually consists of several sub-components.

Another aspect of development that was investigated concerns the regulation of bristle and hair formation. Heat treatment (40.2°C for 40 min) of pupae at different stages resulted in the production of four separate phenocopies designated as angle bristle, smooth bristle, multiple hairs and spear bristle. Each phenocopy is induced at a specific time when the cell is in a susceptible state and the sensitive period lasts for less than two hours.

When animals are heat treated under conditions which do not turn off the protein

synthesis in progress but which induce the heat shock proteins, this results in increased survival and protection against phenocopy production. Comparison of the resumption of protein synthesis to that of RNA synthesis suggests the storage of mRNA as a factor in the protection against phenocopy induction.

Table of Contents

GENERAL INTRODUCTION

One characteristic feature of holometabolous insects is the transformation from larva to adult at metamorphosis. The development of adult features in holometabolous insects varies in the degree of modification of larval features involved. In Neuroptera and Coleoptora, where larvae have some resemblance to adults, relatively little reconstruction occurs. In Diptera, tissues are almost completely rebuilt following histolysis and phagocytosis of the larval tissues. It is generally agreed that phagocytes are not involved in the initial breakdown but only attack the tissue which is in the process of being histolyzed (1).

In Drosophila, the adult stage is preceded by a pupal instar. The pupa is regarded as the equivalent of the last larval instar of hemimetabolous insects and allows the larva to invade entirely new habitats. The actual process of pupation occurs within the hardened puparium 11.5-12 hours after the larva has become quiescent (2). The animal withdraws from the anterior and posterior ends of the puparium by undergoing sudden muscular contractions. These contractions generate a hydrostatic pressure which cause other areas of the body to become fully distended (3). These also lead to the severance of the tracheae and the separation of the prepupal cuticle from the hypoderm. Mitchell (personal communication) observed that the severance of tracheae leaves holes at the ends and at the points of attachment of the tracheoles. This would provide a means for movement of gas and fluids. There is no concrete evidence of diminution of the central gas bubble at the time when the prepupa separates from the puparium.

The pupa lies within three membranes, the newly secreted pupal cuticle, the recently shed prepupal cuticle and the puparium. The prepupal cuticle is essentially a larval cell secretion prior to apolysis (4). Beneath the prepupal cuticle, the cells prepare to secrete the pupal cuticle. The pupal epidermis is a mosaic of larval and

imaginal cells (5) and secretion of the pupal cuticle commences at 3-4 hours after puparium formation. At 12 hours after puparium formation, the one micron thick cuticle is complete.

In insects, as in other animals, protein synthesis is one major biochemical event resulting from cellular activation. The metabolic states and cellular conditions in animals of each species are remarkably specialized. Proteins are produced in homologous tissues and organs and the activation processes leading to the initiation of protein synthesis appears to be species-specific (6).

During metamorphosis, it is conceivable that there is a selective qualitative control of protein synthesis through a coordinated switching of different sets of genes. In general, the capacity for protein synthesis can be inferred at the RNA level which in turn reflects gene activity involved in the morphogenetic process. Studies on nucleic acid and protein metabolism in insects usually have been restricted to certain stages and only a few covered the whole life cycle. In Tribolium confusum, Devi et. al. (7) showed that ^{14}C uridine incorporation into RNA increases during early larval growth and reaches a maximum shortly before pupation. There is a considerable drop at pupation and then it rises slowly again. A nearly identical pattern was found for incorporation of labeled leucine into protein. RNA synthesis precedes protein synthesis at the growth period.

Studies done by Lang et al. (8) in the mosquito, Aedeis aegypti, suggests that the capacity for protein synthesis per cell declines during the later half of larval development. The same picture also holds for developing Calliphora larvae (9).

Experiments have been performed to show that protein synthesis is closely related to the morphogenetic state of the pupa. In 1932, Heller (10) did an extensive analysis of the hemolymph of the lepidopteran, Deilephila euphorbiae, and noted that the larval hemolymph may serve as an important source for the formation of the adult proteins. In Phormia, the hemolymph volume decreases both at the time of pupation and at adult emergence and is accompanied by a rapid fall in protein

concentration (11). These are apparently taken up by tissues for use during adult differentiation as shown by labeled hemolymph injections. In Calliphora stygia, the amount of protein from the hemolymph also decreases at pupation (12). In Drosophila, Boyd and Mitchell (13) observed that some hemolymph proteins had a half-life of only 13 hours whereas other proteins were too stable for their half-life to be determined.

At puparium formation, the lumen of the salivary gland in Drosophila melanogaster becomes extremely narrow. Shortly after puparium formation, the diameter of the gland lumen gradually increases until just before head eversion when the lumen becomes narrow again (14). Von Gaudecker (15) interpreted these observations as a sign of secretory activity of the salivary gland during the period between puparium formation and head eversion.

Many histological studies of the salivary glands of Drosophila noted the accumulation in the cytoplasm of secretory granules which have been assigned various functions, i.e., digestive, food storage, precursor of chitin or as evidence of histolytic changes (16, 17, 18, 19). The true nature and significance of the secretion of the late larval salivary gland was demonstrated by Fraenkel and Brookes (20) when they showed that the secretion is stored in the lumen of the salivary glands until puparium formation when it is expectorated and serves as a glue to affix the puparium to its substrate. A biochemical study of the secretion by Kodani (21) characterized the glue as a mucoprotein with a composition of 70% amino acid and 30% sugar. Korge (22) has also shown that one of the functions of the salivary glands is the secretion of a mucoprotein-containing saliva which makes up 30% of the total protein content of the gland at prepupal formation. This glue allows for the attachment of the pupal case to solid surfaces (14, 22, 23). Fraenkel and Brookes (20) also noted that the secretion of glue does not occur with animals which do not attach their puparium.

The salivary glands of many other families of Diptera are also involved in

the production of one major secretion. The function of the secretion is varied, i.e., tube-building and feeding (e.g., Chironomidae) or slime and cocoon formation (e.g., Mycetophilidae, Cecidomiidae). Following the relase of "PAS-positive" material shortly before puparium formation, the glands of some, if not all, species seem to acquire a new function. Sub-microscopic analysis of prepupal glands of Drosophila melanogaster indicated that the glands become active once again with the synthesis and secretion of one or more substances (24). This is in contrast with previous suggestions that the glands are histolyzed immediately after puparium formation (25, 26). Evidence that the prepupal salivary glands has a secretory function comes from the discovery by Whitten (27) that in Sarcophaga bullata, the prepupal glands accumulate a yellow-brown oily secretion which empties from the lumen at head eversion. The secretion apparently is transferred to the midgut where it surrounds the yellow body. Other investigations which support the secretory role of the salivary glands come from studies of Mitchell et al. (28) which showed that salivary secretions are deposited external to the imaginal hypoderm but inside the thin prepupal cuticle during the prepupal stages.

Chapter 1 of this thesis makes a detailed analysis of the changes in the proteins found in the salivary glands from late larval stage to pupation. Evidence showing rapid rates of synthesis through prepupal development is presented along with data which suggest transport of salivary gland proteins to the space between the thin prepupal cuticle and pupal cuticle at the time of pupation.

Interest in the study of puffs came in 1952 following the paper of Beermann (29), Pavan and Breuer (30) and Mechelke (31). None was concerning Drosophila until the paper of Becker (32) which showed dramatic changes in puffing activity during development in Drosophila melanogaster. Molecular events occurring at the chromosomal level when a band is transformed to a puff is unknown. Presumably, it is regulated by molecules which recognize specific DNA sequences which are contained in specific bands or interbands. Regulation of puffing activity could also

be due to modifications of chromosomal material which allows transcription to be initiated. Studies done by Mitchell and Lipps (33) demonstrated that protein accumulation occurs at a specific puff site just an hour before it is due to puff. Tissières et al. (34) subsequently showed that when glands are heated at 37°C for 20 minutes, a modification of the protein pattern in the salivary glands occurs along with changes in the puffing pattern. These results have been confirmed in both Drosophila melanogaster and D. hydei. In order to gain more information on the roles of specific proteins in the induction or regression of puffs, Mitchell and Lipps (33) compared the protein in nuclei and whole cells of salivary glands with and without heat shock. The protein patterns are identical for the nuclei and whole cells of heat-shocked animals but different in the non-heat shocked animals. These results could be attributed to the induction of new puffs and regression of existing ones or they could also be due to a rapid exchange of proteins between the nucleus and cytoplasm.

It was postulated that heat shock induces phenocopies by interfering with transcription (35). Several investigators (34, 36, 37, 38, 39, 40) have presented evidence that heat shock causes induction of transcriptional activity at a small number of specific chromosomal loci and results in an extreme reduction of RNA synthesis and in regression of transcription at chromosomal loci which are active at the time of the heat shock. Experiments described in Chapters 2 and 3 of this thesis utilize heat shock to gain a better understanding of the molecular basis of phenocopy production. Chapter 2 deals with the phenomenon of inducing bristle and hair phenocopies by the application of a mild heat treatment during a period in the pupal development when the lethal effects of heat shock are minimal but the need for structural proteins is high. Chapter 3 describes experiments which result in increased survival and protection against phenocopy induction by the application of a mild heat treatment prior to the application of a lethal heat shock.

It is largely from a study of puffing activity in the salivary gland polytene chromosomes that the general features of the developmental changes correlated

with puffing in Drosophila are well known. Three major studies of the developmental changes in puffing in Drosophila melanogaster have been published (32, 41, 42). Despite the fact that these three main studies were under different conditions with different strains, there is very good agreement concerning the main features of the puffing patterns. Ashburner (41) noted that there are two major peaks in puffing activity. The first is at the time of puparium formation and the second in 8 hour old prepupae.

One major morphological event occurring in the salivary gland during the period when puffs are studied is the synthesis and secretion of glue glycoprotein. Its period of synthesis correlates with the presence of intermolt puffs in the polytene chromosomes of the salivary gland (43). By recombination and cytogenetic mapping, as well as gene dosage studies, the structural genes for four of the polypeptides were located in intermolt puffs 68C and 3C. Puffs are doubtlessly concerned with manifold aspects of cellular and tissue function. It would be interesting to take a closer examination of some aspects of the developmental physiology of puffs, particularly the striking increase in puffing activity that occurs during the molts and prior to metamorphosis. It would, therefore, be logical to try to correlate the low molecular weight salivary gland proteins analyzed in Chapter 1 to developmentally specific puffs. Becker had recognized 15 puffing stages with puparium formation occurring at PS10-11 and Ashburner extended this analysis to cover the complete prepupal period and described 21 puffing stages, of which 15 were almost identical to Becker (44).

For this study, analyses are directed to puffs wherein the periods of induction and regression coincide with the patterns of protein synthesis obtained from the salivary glands in the late larval to pupal stages. Among the more probable puff sites are 3C, 27C, 28A and 99E. Preliminary studies directed towards addressing this question are included in the Appendix of this thesis.

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Chapter 1

Salivary Gland Proteins and their Role in Pupation

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INTRODUCTION

The functions of the salivary glands of Drosophila melanogaster have been associated, for the most part, with the production of the massive secretion that is expectorated at puparium formation. This so-called glue is proteinaceous and has received extensive attention in relation to regulation of gene activities at specific loci on the salivary gland chromosomes (1-4). However, this function of salivary glands is by no means the only one. As shown by Mitchell et al. (14) the salivary glands go through a series of at least five secretory periods after puparium formation. This conclusion was based on observations of cell structure and was confirmed decisively by Tissières et al. (21). It was demonstrated that salivary glands at all stages from puparium formation to several hours after pupation (about 14 hours) incorporated ^{35}S methionine at about the same rate. This showed clearly a continuous function of the glands. The fact that the patterns of proteins being synthesized throughout the period changed extensively demonstrated changing functions. From these results there seems no doubt that prepupal salivary glands have a variety of functions and that they do not simply undergo a slow histolysis shortly after puparium formation as was suggested by early investigations (5, 20).

The degree of polyteny of salivary gland chromosomes in Drosophila melanogaster has made it possible to study the puffing pattern during the late larval and prepupal stages. Detailed observations by Ashburner (1) showed that the formation and regression of puffs follow a certain sequence and two major peaks of puffing activity were noted. The first is at white puparium formation and the second occurs just before pupation. Experiments done by Tissières et al. (21) indicated a correlation between changes in the pattern of protein synthesis induced by heat shock and modifications in the puff distribution. Furthermore, evidence of control of protein synthesis at the transcriptional level was also presented. Mitchell and Lipps (13) confirmed the conclusion of Berendes (3) that protein accumulation precedes RNA synthesis at

a puff site. Specifically, at 0 hour prepupae pulse-labeled salivary glands showed an accumulation of label at 85D just about an hour before it is due to puff.

In cases where chromosome polyteny is insufficient to observe puffing, changes in translation products were used to measure transcriptional changes. One such case involves translation products related to cuticle formation. Mitchell et al. (15) detected an overlap of the functional salivary gland (terminating at 13 hours) with the completion of the thoracic hypoderm at 8 hours. This suggested that during this period (8 to 13 hrs) salivary secretions can be deposited external to the imaginal hypoderm but inside the thin prepupal cuticle. Interestingly enough, an 80K component which is predominantly produced in the salivary gland is present between these two layers.

Thus, it is reasonable to assume that the salivary gland is a tissue which undergoes significant biochemical changes during development from the late larval to pupal stages. This paper deals specifically with the period from white puparium formation to pupation. The experiments described in this paper show the pattern of activity in the salivary glands with regards to protein synthesis, turnover and transport of certain components to the fluid found between the pupal case and the prepupal cuticle (pupation fluid). Particular attention is focused on lower molecular weight proteins (proteins a, b, c and d) because of their very high levels of incorporation of labeled amino acids and subsequent presence in the pupation fluid.

MATERIALS AND METHODS

Fly Culture

Oregon-R wild stock of Drosophila melanogaster were raised in mass culture at 25°C as described by Mitchell and Mitchell (12). The animals were collected at the late larval stage and white prepupae selected when puparium formation started one to two hours later. Subsequent timed samples were taken every hour past puparium

formation. For some experiments, 5-6 hour prepupae were selected by the flotation method (12).

Labeling

Five pairs of salivary glands were dissected at regular time periods from late larval stage to pupal stage and labeled with ^{35}S methionine or ^{35}S cysteine. Labeling was done by incubating the glands in 5 µl of "MOPS" buffer (80 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 10 mM MOPS and adjusted to pH 7.0 with NaOH) containing 10 µCi of ^{35}S methionine (sp. act. 1105.6 Ci/mmol, New England Nuclear) or 10 µCi of ^{35}S cysteine (sp. act. 984 Ci/mmole, New England Nuclear) for 20 minutes at room temperature. The incubation mixture was diluted by adding "MOPS" 30-40 times the volume of the reaction mixture. The glands were then transferred from the label to a conical centrifuge tube (5 mm x 75 mm) containing 30 µl of SDS sample buffer (21) with about 0.001% bromphenol blue. The sample buffer was modified to contain dithiothreitol at a concentration of 0.2% instead of 1% B-mercapto-ethanol. Samples were heated in a boiling water bath for 5 minutes and kept at -70°C until just before electrophoresis.

Injection

Animals were collected at the white prepupal stage and kept at 25°C on a piece of moistened Whatman filter paper kept in a petri dish. Approximately three hours hence, they were lined up on double-faced Scotch tape along one side of a microscope slide. They were subsequently injected with ^{35}S methionine (0.5 mCi/40 µl MOPS). Injections were carried out with an apparatus developed by H. K. Mitchell (16). It is adjustable to deliver solutions in the range of 0.02 to 0.2 µl and at 0.06 µl the volume is reproducible to $\pm$5%. Injection needles were drawn from 1 mm glass tubing and beveled at a tip diameter of approximately 50 microns. Incorporation was allowed to proceed for one hour before four pairs of salivary glands were dissected out and transferred into a conical centrifuge tube containing SDS sample buffer. Each sample was prepared for gel electrophoresis as described.

The pupation fluid was collected by removing the pupal case without puncturing the prepupal cuticle. This is done in approximately 20 µl of "MOPS" buffer. The case is left in the buffer for a few minutes and the buffer collected into a conical centrifuge tube. The protein is precipitated by adding TCA to a concentration of 10%, washed twice with ethanol, and then heated in sample buffer for 5 minutes.

Gel Electrophoresis and Autoradiography

Gel electrophoresis was carried out on slab gels (18 cm x 22.5 cm) made with a linear gradient of 7.8%-20% acrylamide, 0.1%-2.6% bisacrylamide. The SDS-containing buffers of Laemmli (10) were used. The samples were run at room temperature at a constant current of 14 milliamperes for 15 hours. Staining and destaining were done as described by Fairbanks et al. (6). The gels were dried and exposed for autoradiography on KODAK XAR-5 X-ray film. Autoradiograph exposures were usually done in 4-7 days.

Two-Dimensional Gel Electrophoresis

For the analysis of the proteins from the salivary glands and the pupation fluid of injected animals, the first dimension of the two-dimensional gel electrophoresis was carried out as a nonequilibrium isoelectric focusing gel as described by O'Farrell et al. (18) using equal volumes of Pharmalyte 7-9 and Pharmalyte 8-10.5 (Pharmacia Fine Chemicals). Isoelectric focusing was done at room temperature with a constant voltage of 400 volts to a total of 1400 volt-hours. The second dimension was done on an SDS-acrylamide gel as described. The gel was then fixed in 50% methanol, dried, and exposed for autoradiography.

For analysis of the pulse-labeled salivary glands, the first dimension was carried out as an equilibrium isoelectric focusing gel (17) using Pharmalyte 5-8 and 8-10.5 in a ratio of 1:7 respectively. The isoelectric focusing was done at room temperature in constant wattage with a starting voltage of 600 volts and was run for a total of 5000 volt-hours. The second dimension was done in an SDS-polyacrylamide gel electro-

phoresis as described. The gel was then prepared for autoradiography in the same manner as described above.

Densitometer Tracings

Autoradiographs were scanned with a Joyce, Loebl and Company densitometer and the incorporation levels into the proteins of interest were plotted according to their peak heights.

Chromatofocusing

Labeled salivary glands in 2 µl of sample buffer and 100 µl of 0.025M ethanolamine-HCl, pH 9.4 were heated in a boiling water bath for six minutes and applied on a column (2 mm x 12.5 mm) containing an ion exchanger "PBE 94" (Pharmacia Fine Chemicals) which had been equilibrated with 0.025M ethanolamine-HCl, pH 9.4. The sample was eluted with Pharmalyte 8-10.5 adjusted to pH 8.0 with HCl. Column fractions measuring 100 µl were collected and the pH and radioactivities were assayed.

Time-Lapse Photography

5-6 hour prepupae were collected and a pair was lined up on a microscope slide. Animals in developmental synchrony were selected by the flotation method as described by Mitchell and Mitchell (12). Pictures were taken for a period of five hours at a rate of 12 frames per minute with a 1 minute exposure per frame.

RESULTS

Patterns of Activity in the Process of Pupation

At the onset of pupation, the prepupa encounters a high degree of muscular activity in the form of a series of contractions and sideward movements. A detailed sequence of their activities is shown in Fig. 1. At 11-12 hours after puparium formation, the prepupa suddenly contracts away from the posterior end. This contraction takes place within 3-4 minutes and is followed by a series of alternating sideward movements which continue for about 6 minutes. These fast movements cause the tracheae to

break at the posterior end. The animal then squirms and retreats to the posterior end within the next 10 minutes. The next wave of contractions and sideward movements then follows and continues for 2 minutes. Finally, during the subsequent 18 minutes, the legs evert and the head emerges from the thorax. The end of pupation is marked by the movement of the prepupa towards the posterior end and the pupa remains quiescent until the stage (3.5 days later) when it emerges from the puparium. A similar sequence of events has been observed by Robertson (19) and Fristrom (7).

Protein Patterns through Development

The most abundant proteins present in salivary glands at different stages are shown in Fig. 2a as bands from staining with Coomassie blue. Those proteins which are being synthesized most rapidly at a given time are shown in Fig. 2b by autoradiographs from pulse-labeling. In both cases attention is drawn especially to components labeled a, b, c and d since these appear to be the ones most involved in the pupation process.

In Fig. 2a, many of the predominant proteins detectable by Coomassie stain are present in all stages from late larvae to pupae. Among the strongly visible proteins, 32 are present from late larval to pupation stages with quantitative differences in various stages. In general, the proteins are present in maximum amounts at 5 hours after puparium formation, especially for 8 proteins (36 mm, 38 mm, 62 mm, 65 mm, 87 mm, 103 mm, 143 mm, 150 mm). Two of these 8 proteins (87 mm, 103 mm) are present at equivalent levels during the late larval stage but drastically diminish at puparium formation. A gradual accumulation follows up to 5 hours after puparium formation and then diminution occurs again to the same extent as after the late larval stage. The band at 129 mm gradually increases and peaks at 7-8 hours after pupariation and then it diminishes drastically at pupation. The protein at 143 mm also shows the same pattern of accumulation until 5 hours after pupariation and then gradually decreases until just before pupation. At pupation, the intensity of

the band shows a notable increase. Protein a which bands at 137 mm remains constant during development from late larvae to pupae. Protein b accumulates until 5 hours after puparium formation but considerably decreases afterwards and stays at the reduced level even after pupation. Protein c shows accumulation from the late larval stage and through the different prepupal stages. A significant reduction then occurs at pupation. In the case of protein d, the accumulation which takes place through the different prepupal stages leads to a prominent increase in intensity at the time of pupation. Thus, a majority of the most abundant proteins are present at the same level from prepupal to pupal stages but the small molecular weight-proteins certainly exhibit changes in concentration through the different developmental stages.

The autoradiograph of the gel in Fig. 2a is shown in Fig. 2b. This shows extensive changes in the patterns of proteins being synthesized based on the level of incorporation of labeled amino acid. At the start of puparium formation and 1 hour hence, no syntheses of new proteins occurs and the protein at 118 mm is synthesized only at the late larval stage. At 2 hours after puparium formation, more synthesis takes place and the levels of incorporation are appreciably higher for proteins a, b and d. When salivary glands are pulse-labeled with cysteine (Fig. 3) some components appear more abundant at the late larval stage compared to the other stages (i.e., 71 mm, 92 mm, 117 mm). Among the 32 proteins visible on the autoradiography, the levels of syntheses for the larger proteins seem constant all the way to 8 hours after pupariation with some discrete changes in the intensity (i.e., 44 mm, 62 mm). However, the rates of synthesis for the smaller proteins are definitely greater and they progressively increase to a maximum at 5-6 hours after puparium formation. At pupation, there is either an increase in the levels of syntheses among some of the higher molecular weight components (i.e., 45 mm, 80 mm, 96 mm, 99 mm, 103 mm and 122 mm) or no change at all. In contrast, a drastic decrease in the syntheses of proteins a-d and the proteins at 157 mm and 160 mm occur at the time of pupation.

When salivary glands are pulse-labeled with cysteine (Fig. 3) some components appear more abundant at the late larval stage compared to the other stages. Proteins a-d correspond to a-d in Fig. 2. Similarly, the proteins in the low molecular weight region show more variability through development and are all present in reduced levels when pupation occurs. The autoradiograph in Fig. 3b shows that synthesis, measured by amino acid incorporation, occurs at an extremely low level at puparium formation and gradually increases until 8 hours after pupariation. At 1 hour past puparium formation, a majority of the proteins being synthesized comprise a different set compared to those present during the late larval stage. The phenomenon of very high levels of incorporation among the smaller polypeptides is still maintained. When pupation occurs, the low molecular weight species show a drastic reduction in the levels of incorporation of cysteine compared to the high molecular weight proteins. This is similar to what was observed in methionine-labeled glands. Another point of similarity is the differential periods of synthesis for these polypeptides. In the late larval stage, the rates of syntheses for the proteins at 71 mm and 92 mm are quite low whereas the protein at 117 mm shows a very high rate of synthesis. Synthesis is hardly detectable at puparium formation but increases to a detectable level an hour later. However, the proteins at 110 mm and 125 mm still show less label at this stage compared to the other components. Proteins a, b and d are being synthesized at 1 hour after puparium formation whereas synthesis of protein c is not detectable until 4 hours after puparium formation. At 5 hours after puparium formation, the synthesis of protein a is drastically diminished whereas proteins b, c and d still show increasingly high levels of incorporation up to 8 hours after puparium formation (Fig. 4).

It should be noted that some of the proteins incorporate methionine and cysteine at equivalent levels (proteins a, b and c—Figs. 2b vs. 3b) whereas other proteins incorporate more methionine than cysteine (Protein d—Figs. 2b vs. 3b) and vice

versa (135 mm—Figs. 2b vs. 3b). This was determined by pulse-labeling salivary glands with labeled methionine or labeled cysteine and analyzing the protein patterns obtained under identical experimental conditions (Fig. 5).

The preceding data show that the salivary glands are constantly synthesizing a considerable number of polypeptides during the prepupal stages. The synthesis is proportionally low at puparium formation but quickly recovers an hour or two later. Particularly interesting is the enhanced level of synthesis of the smaller polypeptides during the prepupal stages and their extreme reduction at pupation. When visualized with Coomassie, these small polypeptides show a pattern of being accumulated up to a certain stage and then they are utilized.

Protein Turnover

In order to ascertain whether there is rapid synthesis and turnover of salivary gland proteins through the different developmental stages leading to pupation, prepupae were injected with labeled methionine at 3-4 hours after puparium formation and protein patterns were analyzed from glands dissected at the stages indicated in Fig. 6. Fig. 6a shows the Coomassie-stained patterns of the proteins through the prepupal stages. Results suggest a general maintenance of most, if not all, of the proteins present except for the low molecular weight ones which diminish drastically at pupation. Proteins a-d in Fig. 6 are equivalent to proteins a-d in the pulse-label experiments with respect to their mobilities (Figs. 2 and 3).

The autoradiograph in Fig. 6b shows a noticeably higher amount of low molecular weight polypeptides present in the prepupal stages. Moreover, the intensity and the number of the labeled bands remain essentially the same through the different prepupal stages. This implies that there is no discernible turnover of these proteins before pupation. A comparison of the pattern of protein synthesis in Fig. 2b with the pattern obtained from salivary glands of injected animals shows that the proteins being synthesized at the time of injection are essentially still present 2-3 hours hence (compare +4 in Fig. 2b to +6 in Fig. 6b). At pupation, an extreme reduction

of these small proteins transpires in the salivary glands and a proportionate increase of these low molecular weight proteins occur in the pupation fluid.

Transport of Salivary Gland Proteins to the Fluid

The injection experiments indicate that the four low molecular weight proteins of interest which are present in the salivary glands are excreted or transported to the pupation fluid at the time of pupation (Fig. 6). An exception to this is protein a which is not detectable in the salivary gland nor in the pupation fluid. To support this hypothesis, it is important to show that the proteins found in the salivary glands and in the pupation fluid are identical. Two-dimensional gel analysis of proteins from the glands and the pupation fluid of injected animals shows that the low molecular weight components are indeed identical (Fig. 7). Furthermore, each component with a specific molecular weight consists of 3-4 subcomponents with distinct isoelectric points. To illustrate, there are at least 4 components in the region where protein d migrates which have pIs from 8.75-9.9. Conversely, there are 4 components of different molecular weights (corresponding to proteins a, b, c and d) with isoelectric points of 8.75. Therefore, what is detected as a single component in one dimensional analysis is actually a representation of several polypeptides. The isoelectric points are approximate values since the focusing system used is a nonequilibrium isoelectric focusing due to the high basicity of the proteins. Pulse-labeled glands also show the same pattern of proteins on a two-dimensional gel electrophoresis which confirms that the components detected in the pulse-label experiments are similar to what are obtained from the injected animals.

Chromatofocusing was done in order to get a more accurate isoelectric point of the proteins. Fig. 8 shows the isoelectric point profile for the proteins in the salivary glands and in the pupation fluid. The results confirm that these polypeptides are highly basic proteins. In both samples, the major components have isoelectric points of about 8.7 and the minor peak is approximately 9.2. The combined properties

of high basicity and low molecular weight augmented by interference by the ampholyte account for the difficulty in getting a better resolution of these polypeptides in a two-dimensional gel.

DISCUSSION

In Drosophila melanogaster, puparium formation occurs approximately 120 hours after egg-laying and pupation takes place 11-12 hours after puparium formation. Time-lapse pictures taken from 5 hours after puparium formation (or white prepupal stage) to pupation show a series of muscular contractions which lead to severance of tracheae and mouth parts with accompanying head invagination. Robertson (19) proposed that the contractions probably effects a separation of the prepupal cuticle from the hypodermis and results in a partial withdrawal of the tracheal tubes attached to the posterior spiracles. Fristrom (7) noted that the alternating sideward contractions effect the movement of gas from the posterior to the anterior region of the pupa and account for the detachment of the posterior spiracles. The gradual movement of the air bubble gradually forces the pupa backwards until the attachments to the larval mouth part and to the tracheae are severed. This is followed by the emergence of the head from the thorax and subsequent displacement of the pupa to the rear of the case.

This work describes an analysis of the proteins present in the salivary glands during development from late larvae to pupae. Data are based mainly on in vitro pulse-labeling of the salivary glands with ^{35}S methionine or ^{35}S cysteine and the rates of synthesis expressed in terms of amino acid incorporation. Labeled amino acids were also injected into white prepupae and the course of disappearance and accumulation followed through development to the pupal stage.

When salivary glands are pulse-labeled with methionine or cysteine, proteins detected by staining with Coomassie blue exhibit prominent changes through prepupal

development. High molecular weight proteins seem to accumulate to a maximum at 5 hours after puparium formation and are then selectively utilized in the subsequent stages. Attention is directed primarily on a set of low molecular weight proteins because of their stage-specific expression and utilization (Fig. 2a). The pattern of synthesis based on amino acid incorporation also indicates that these small polypeptides are being very actively synthesized in the salivary glands. Synthesis is at a minimum during puparium formation and 1 hour hence but the rate rapidly increases at 2 hours after puparium formation. Of the 4 proteins of interest (proteins a-d) only 3 are being synthesized at this stage (a, b, d) and synthesis of the fourth one (c) starts about 3 hours later.

Synthesis reaches a maximum at 5-6 hours after puparium formation and slowly decreases subsequently. However, at pupation, the salivary gland manifests an extreme reduction in the synthesis of the low molecular wieght proteins. When glands from prepupae which had been injected with labeled amino acids are examined, the loss of these specific set of proteins is even more remarkable (Fig. 6). This suggests that these proteins may be secreted by the gland at the time of pupation. Mitchell et al. (14) has shown that the salivary glands function as a secretory organ through the prepupal stage and even after pupation. Histolysis occurs suddenly after pupation rather than gradually over the whole prepupal period. In view of these findings, the fluid between the pupal case and the prepupal cuticle (pupation fluid) was considered as a conceivable site of these secreted products. Analysis of the pupation fluid does show this set of proteins to be a major component of the fluid. Other tissues which survive metamorphosis like the brain and the hemolymph do not show the presence of these small proteins (Fig. 9). Two-dimensional gel electrophoresis (isoelectric focusing in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension) strongly support the hypothesis that these small polypeptides are produced in the salivary glands and are subsequently released/transported to the pupation fluid at the time of pupation.

The data also indicate that each component which is manifested as a single band actually consists of more than one protein. A specific example is protein c in the salivary glands from injected animals (Fig. 6). This component shows up as a strongly stained band at 6 hours after puparium formation and disappears 2 hours later (Fig. 6a). However, in the autoradiograph of the same gel (Fig. 6b) protein c is discernible at 8 hours after puparium formation and is not detectable during the earlier stage. This implies that the band of protein c actually consists of different polypeptides; one is accumulated in the early prepupal stages and diminishes at 8 hours after puparium formation and another is synthesized at 8 hours after puparium formation and is maintained in the salivary gland until the time of pupation when it is significantly reduced.

Results from two-dimensional gel analyses do indicate the presence of more than a single protein in each band. As shown in Fig. 7, protein d consists of at least 4 sub-components, protein c has 3 sub-components and proteins a and b have at least 3 sub-components each.

A property of these small proteins which can be inferred from pulse-labeling with cysteine or methionine is that some of these polypeptides are methionine-rich and others are cysteine-rich. Specifically, a single band in the SDS-polyacrylamide gel has different levels of incorporation for methionine and cysteine (Fig. 5). For example, protein d incorporates more methionine than cysteine whereas the band at 135 mm (Fig. 2b vs. 3b) selectively incorporates cysteine over methionine. Another property of these proteins is their high basicity. A major portion of the low molecular weight proteins have isoelectric points of 8.6-8.7 and a minor portion of the population has isoelectric points in the range of 9.1-9.5 (Fig. 8).

We can only speculate about the possible functions of these small polypeptides. One probable function is its enzymatic activity because certain abdominal muscles do undergo slight histolysis at pupation. Its presence in the fluid which exists between

the prepupal and the pupal cuticle also suggests the possibility of some chitinase activity. These small polypeptides could also be secreted proteins which serve as a lubricant in order to allow the animal to undergo the muscular movements required for pupation. Treatment with tunicamycin did not inhibit the presence of these small polypeptides, hence it might be unlikely that they are glycosylated proteins.

Fukuda (8) showed that the prothoracic gland of silkworm releases into the blood active principles responsible for the onset of pupation. Ligation of the posterior segment of the prothoracic gland prevents pupation whereas ligation of the anterior including the prothoracic segment enables pupation. When this was done on older larva after it had spun the greater part of its cocoon, the posterior part without the prothoracic segment pupated. These findings led him to conclude that the prothoracic segment probably represents a center of internal secretion which at a critical period releases into the blood the factors required for pupation. It is, therefore, conceivable that a similar mechanism exists in the salivary glands of Drosophila melanogaster.

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15. Mitchell, H. K., Lipps, L. S. and Tracy, U. W. (1977b). Biochem. Genetics **15**, 575-587.
16. Mitchell, H. K. (1978). In The Genetics and Biology of Drosophila, 2a, M. Ashburner and T. R. F. Wright, eds. (New York: Academic Press) pp. 150-158.
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Fig. 1. Muscular activity in the process of pupation.

5-6 hour prepupae were selected by the flotation method. Time-lapse pictures were taken for 5 hours at a rate of 12 frames per minute with one second exposure per frame. The picture series illustrates activity within the hour when pupation occurs. The first frame was taken 11-12 hours after puparium formation (PP) and subsequent time periods indicated are the minutes which had lapsed since the first activity depicted in the first frame.

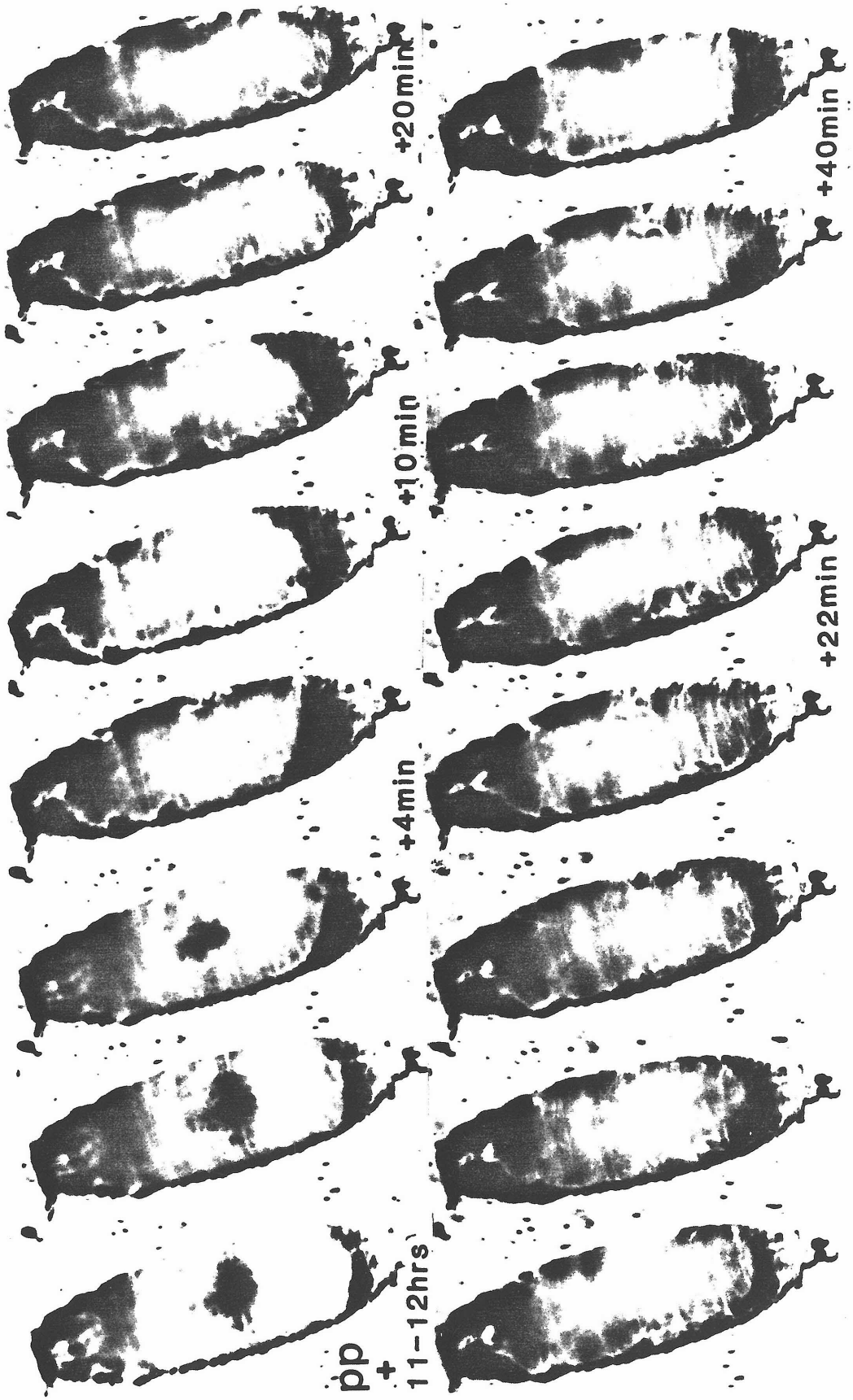

pp
+
11–12hrs
+4min
+10min
+20min
+22min
+40min

Fig. 2. Protein pattern of pulse-labeled salivary glands from the late larval stage to pupation.

Four pairs of salivary glands were labeled with 20 μCi of ^{35}S methionine for 20 minutes at 23°C.

(a) Coomassie-stained pattern of SDS-polyacrylamide gel electrophoresis. The numbers on the left side of the figure indicate the migration distances in millimeters from the origin of the gel. 6.5 K and 3.4 K are molecular weight markers. The small polypeptides of interest which are discussed in the text are referred to as a, b, c, d. Samples were taken from the late larval stage (LL), white prepupal stage (puparium formation) (PP), and the subsequent numbers for each lane refer to the hours after puparium formation when the salivary glands were dissected and pulse-labeled. P+1 refers to the time period when pupation occurs.

(b) Autoradiograph pattern of SDS-polyacrylamide gel electrophoresis of pulse-labeled salivary glands shown in (a). The first two lanes contain radioactive molecular weight markers. The numbers on both sides of the figure are the migration distances from the origin of the gel designated in mm.

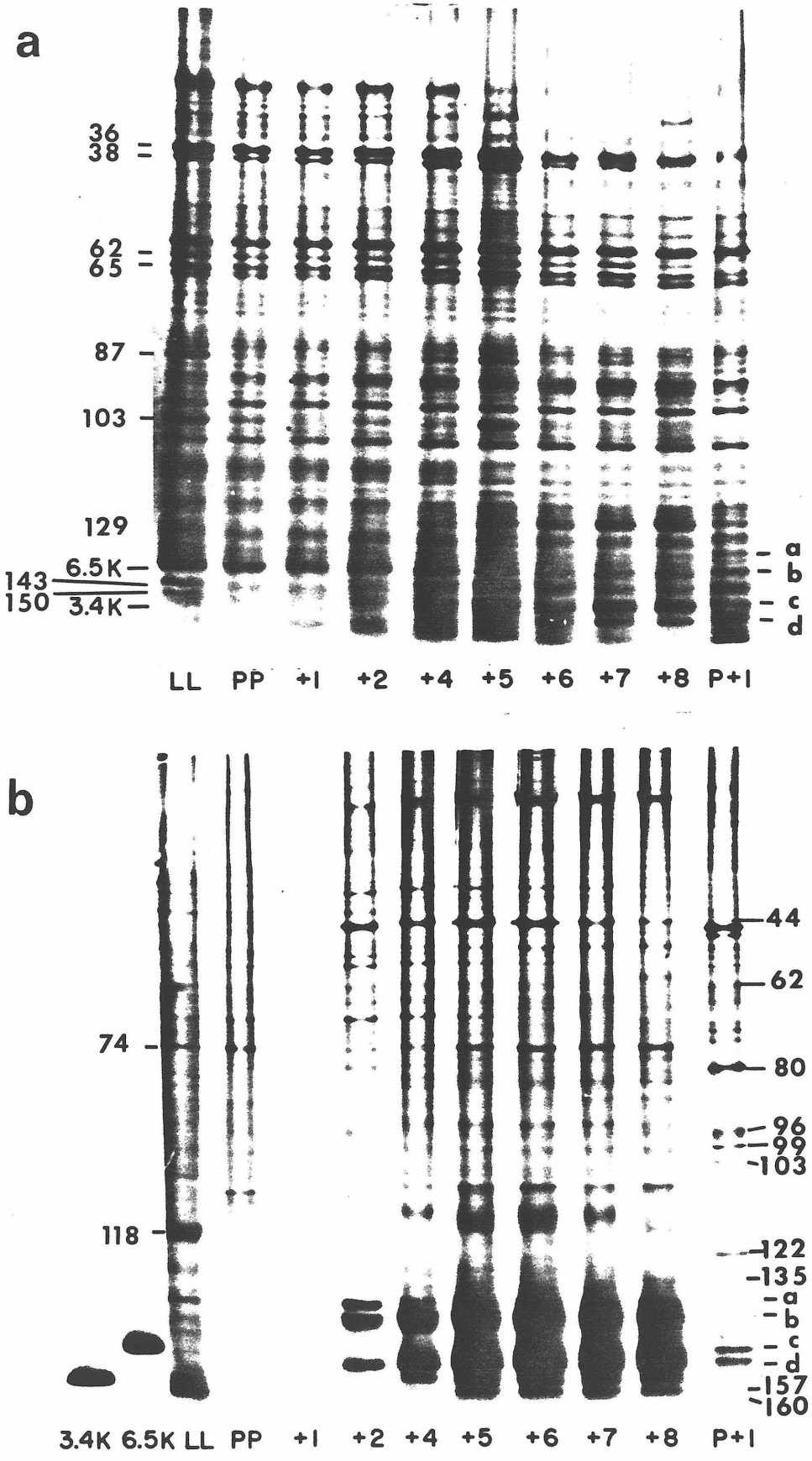

a
36
38
62
65
87
103
129
6.5K
143
150
3.4K
a
b
c
d
LL PP +1 +2 +4 +5 +6 +7 +8 P+1
b
74
118
44
62
80
96
99
103
122
135
a
b
c
d
157
160
3.4K 6.5K LL PP +1 +2 +4 +5 +6 +7 +8 P+1

Fig. 3. Protein pattern of salivary glands from different developmental stages which are pulse-labeled with ^{35}S cysteine.

Stained (a) and autoradiograph (b) patterns of SDS-polyacrylamide gel electrophoresis of salivary glands labeled with 20 μCi of ^{35}S cysteine for 20 minutes at 23°C. The numbers on both sides of the figure indicate the migration distances from the origin of the gel designated in mm. Samples were taken from the late larval stage (LL), at puparium formation (PP) and at periodic intervals after puparium formation as indicated by the numbers of hours shown for each lane. P+1 refers to the time period when pupation occurs. The low molecular weight proteins are designated a-d and correspond to the proteins a-d demonstrated in Fig. 2.

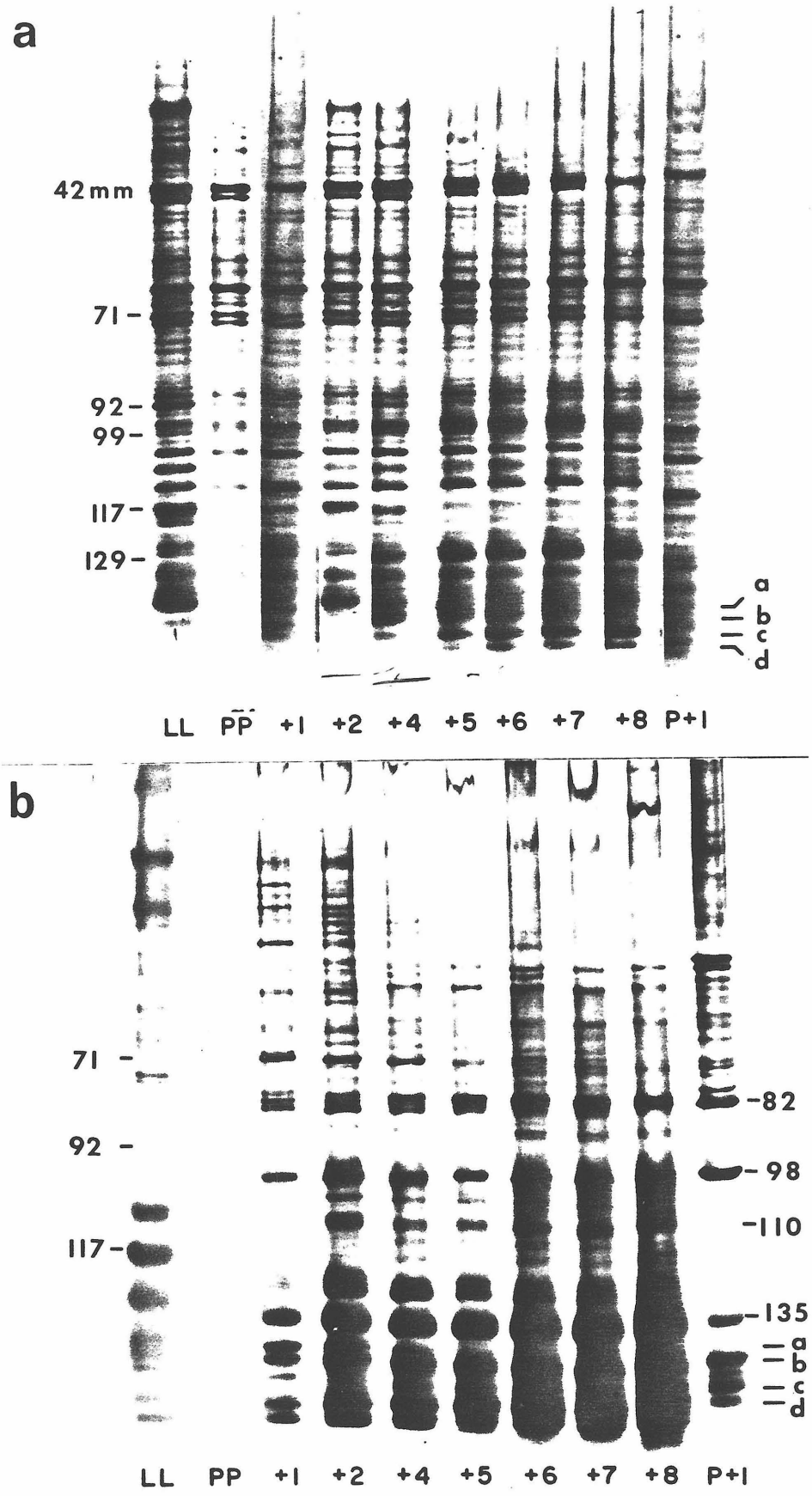
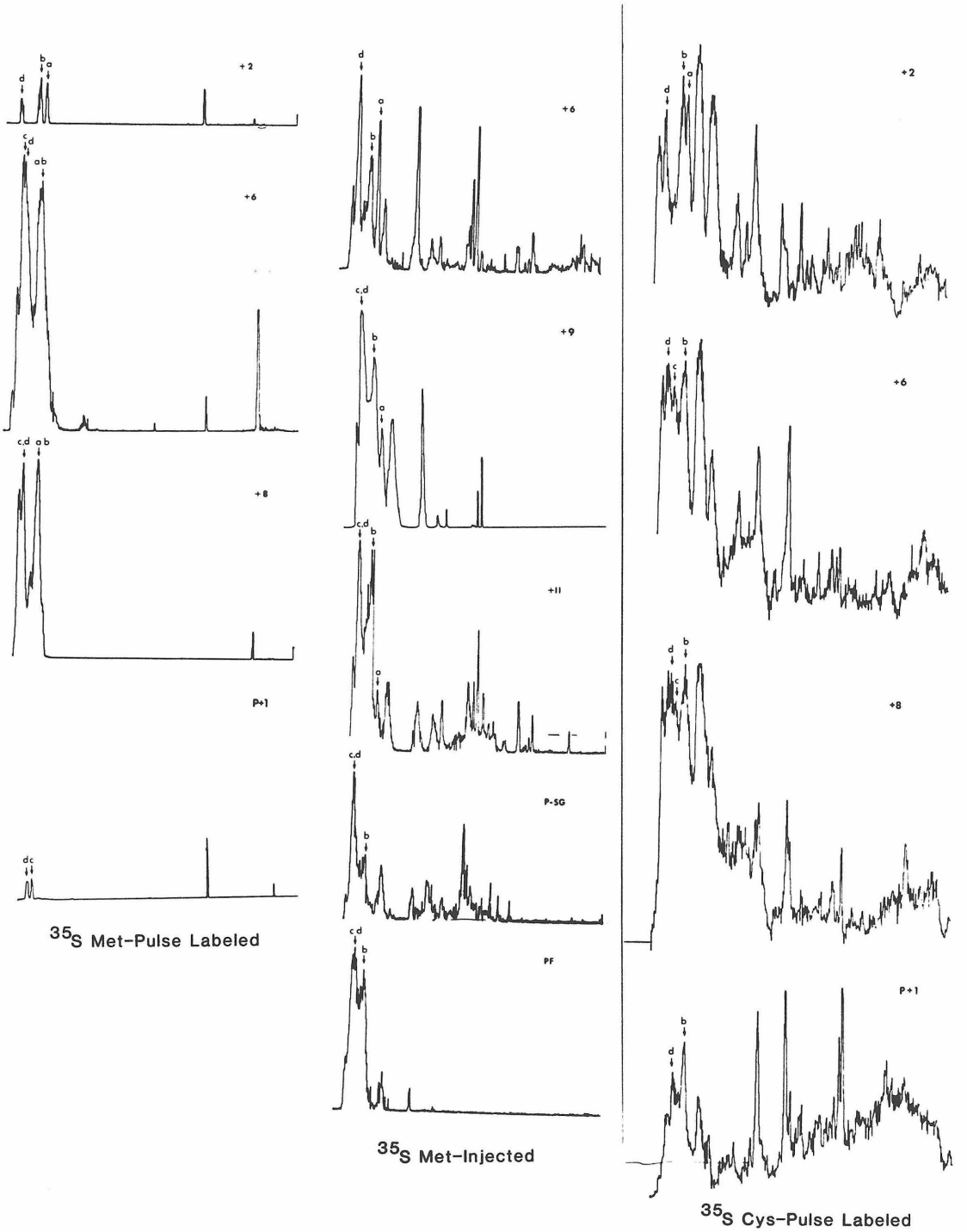

a
42mm
71
92
99
117
129
a
b
c
d
LL PP +1 +2 +4 +5 +6 +7 +8 P+1
b
71
92
117
82
98
110
135
a
b
c
d
LL PP +1 +2 +4 +5 +6 +7 +8 P+1

Fig. 4. Graphical representation of the rates of syntheses of proteins a-d.

Densitometer tracings of autoradiograms (a) and a graph of the densitometer tracings according to their heights (b) were done on specified samples from Figs. 2, 3 and 6.


+2
+6
+8
P+1
35S Met-Pulse Labeled
+6
+9
+11
P-SG
PF
35S Met-Injected
+2
+6
+8
P+1
35S Cys-Pulse Labeled

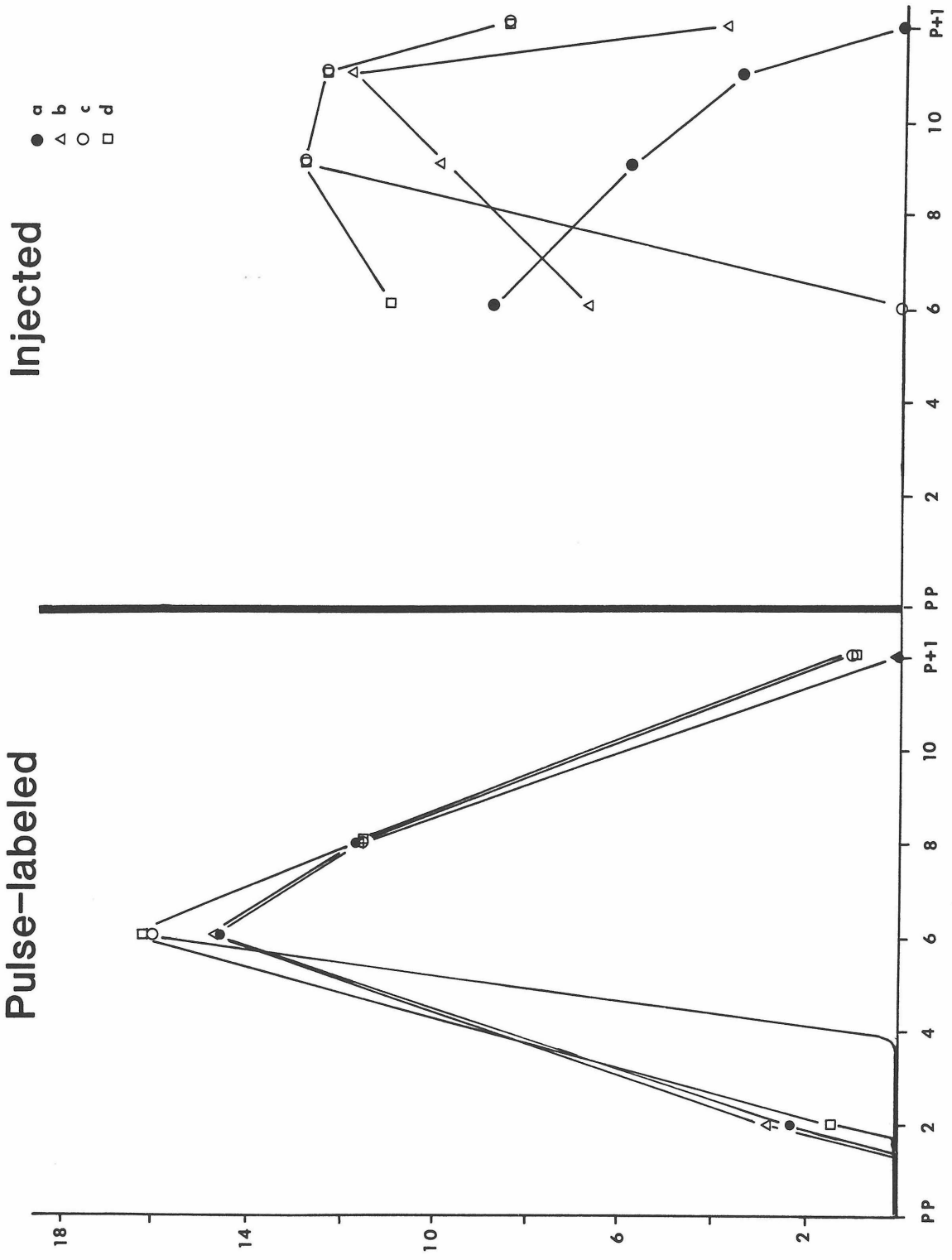

Pulse-labeled
Injected
a
b
c
d
18
14
10
6
2
PP
2
4
6
8
10
P+1
PP
2
4
6
8
10
P+1

Fig. 5. Comparison of the levels of incorporation for methionine and cysteine.

Autoradiograph pattern of salivary glands pulse-labeled with ^{35}S methionine or ^{35}S cysteine. Five pairs of salivary glands were labeled either with 20 μCi of ^{35}S methionine or 20 μCi cysteine for 20 minutes at 23°C. The numbers below each lane represent the number of hours past puparium formation when the samples were taken. The two lanes on the left side show the pattern obtained from glands labeled with methionine and the two lanes on the right side represent the patterns obtained from glands labeled with cysteine.

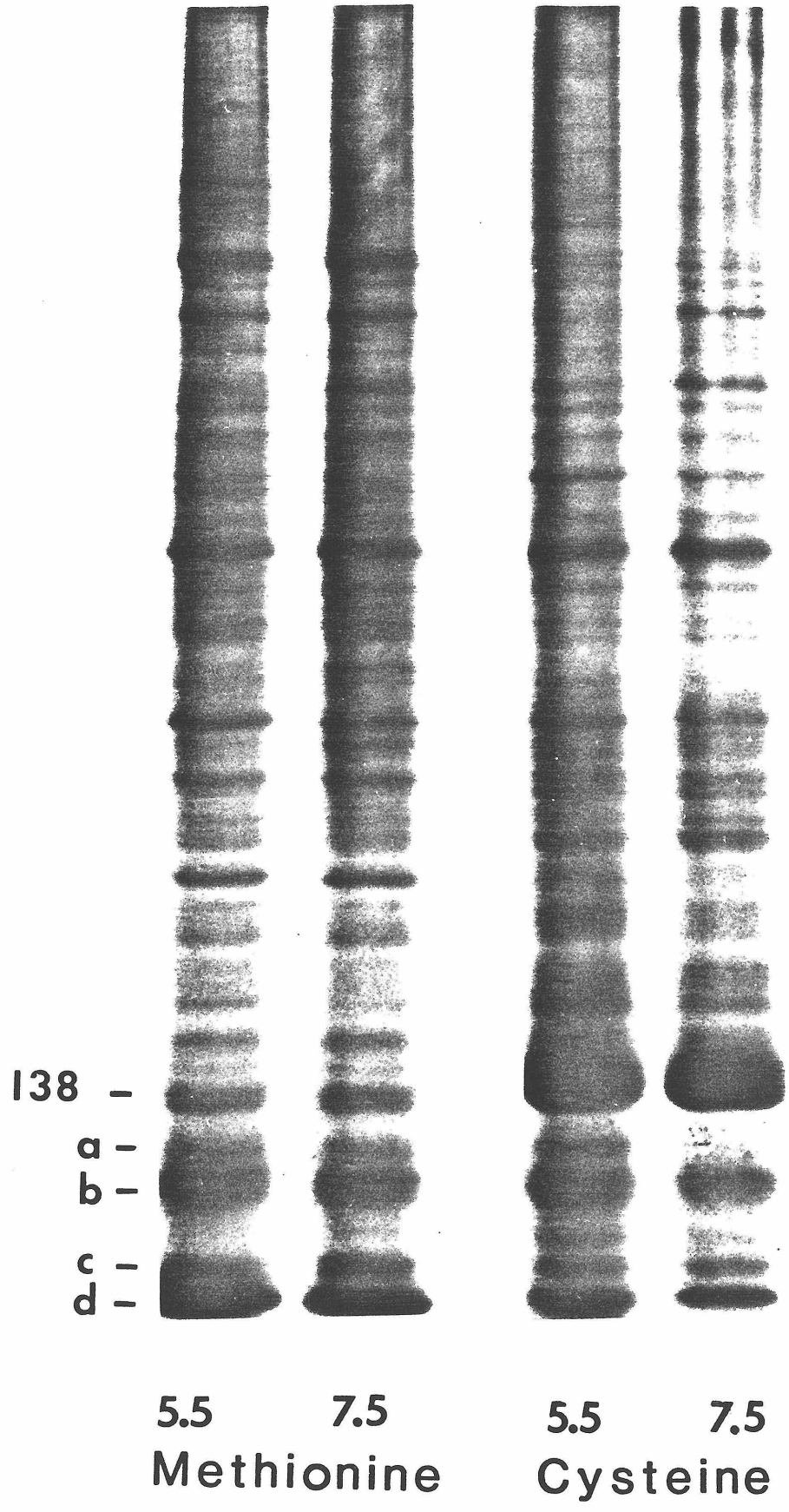

138
a
b
c
d
5.5
7.5
Methionine
5.5
7.5
Cysteine

Fig. 6. Analysis of proteins in the salivary glands of animals injected with labeled methionine.

Coomassie stained (a) and autoradiograph (b) patterns of proteins from salivary glands of prepupae injected with ^{35}S methionine. 3-4 hour prepupae were injected with ^{35}S methionine (0.5 mCi/40 µl MOPS) and incorporation was allowed to proceed for one hour. Salivary glands were dissected at specific time periods after puparium formation as indicated on each lane. SG and PF refer to salivary gland and pupation fluid taken when pupation occurs. Samples on the last two lanes of 6(b) are radioactive molecular weight markers, namely, aprotinin and cytochrome c. a-d are the low molecular polypeptides of interest discussed in the text and correspond to the same proteins indicated in Figs. 2-4.

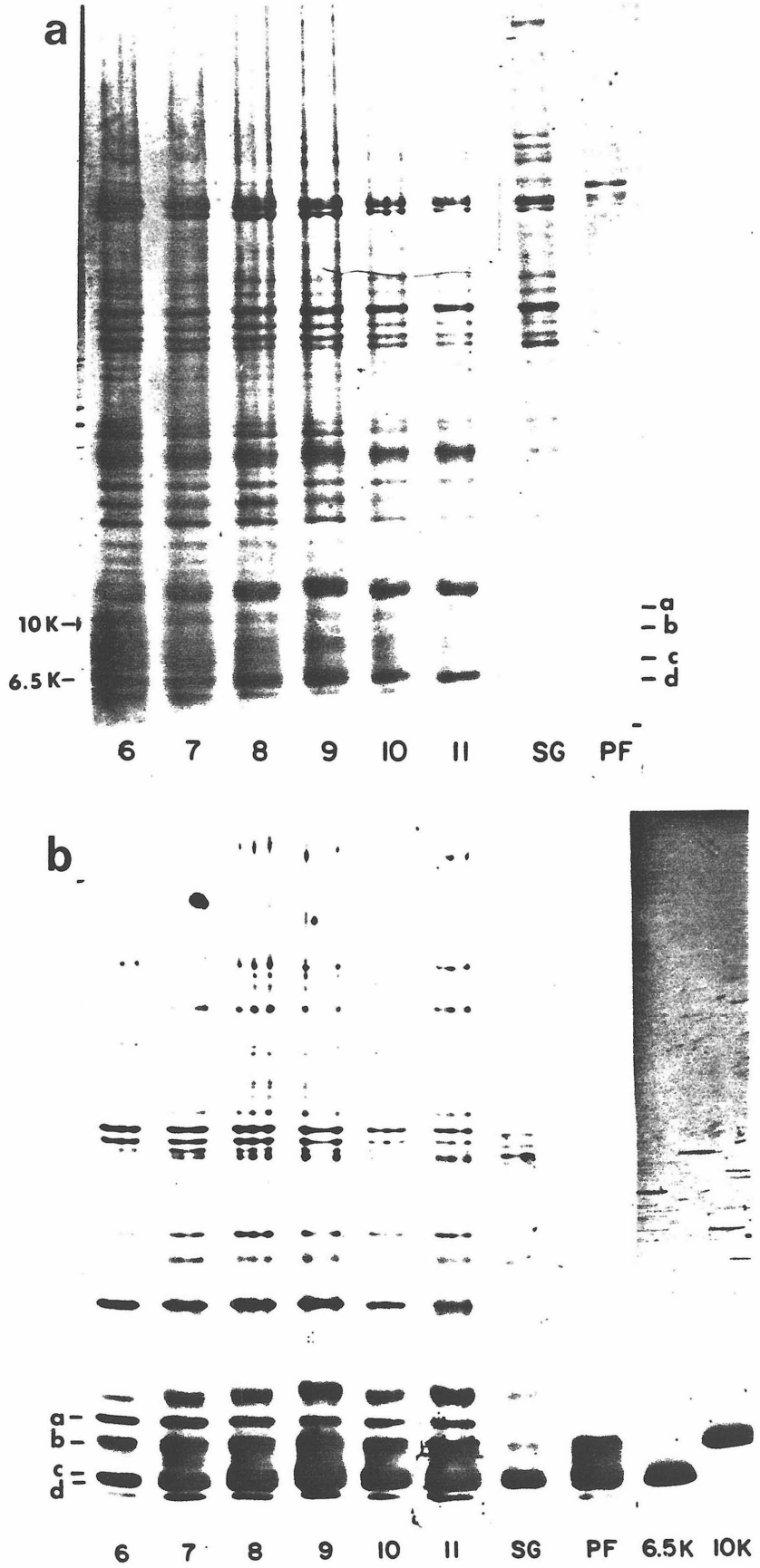

a
10K
6.5K
a
b
c
d
6 7 8 9 10 11 SG PF
b
a
b
c
d
6 7 8 9 10 11 SG PF 6.5K 10K

Fig. 7. Comparison of the two-dimensional gel electrophoretic pattern of proteins from the salivary glands and pupation fluid of injected animals with the proteins of pulse-labeled salivary glands.

(a) Two-dimensional gel electrophoresis of pulse-labeled salivary glands from 8-9 hour prepupae. 3 pairs of salivary glands were pulse-labeled with ^{35}S methionine. The first dimension proceeds from right to left and is an equilibrium isoelectric focusing using Pharmalyte 5-8 and 8-10.5 in a ratio of 1:7 respectively. The isoelectric focusing was done at room temperature in constant wattage with a starting voltage of 600 volts to a total of 5000 volt-hours. The second dimension is a SDS-polyacrylamide gel electrophoresis with a linear gradient of 7.8-20% acrylamide and 0.1-2.6% bisacrylamide run at room temperature with a constant current of 14 milliamperes for 15 hours.

(b) Two-dimensional gel electrophoresis of proteins from the salivary glands and the pupation fluid of prepupae injected with ^{35}S methionine (0.2 mCi/15 µl MOPS) and ^{35}S cysteine (0.3 mCi/15 µl MOPS). 25 pairs of salivary glands and pupation fluid from 49 animals were collected at the time of pupation. The first dimension proceeds from right to left and is a nonequilibrium isoelectric focusing using equal volumes of Pharmalyte 7-9 and 8-10.5. The isoelectric focusing was done at room temperature with a constant voltage of 400 volts to a total of 1400 volt-hours. The second dimension is a SDS-polyacrylamide gel electrophoresis (linear gradient of 7.8-20% acrylamide; 0.1-2.6% bisacrylamide) run at room temperature with a constant current of 14 milliamperes for 15 hours. The center lane is from a sample consisting of 22 pairs of labeled salivary glands taken at the time of pupation and is used as a marker for the proteins in the second dimension.

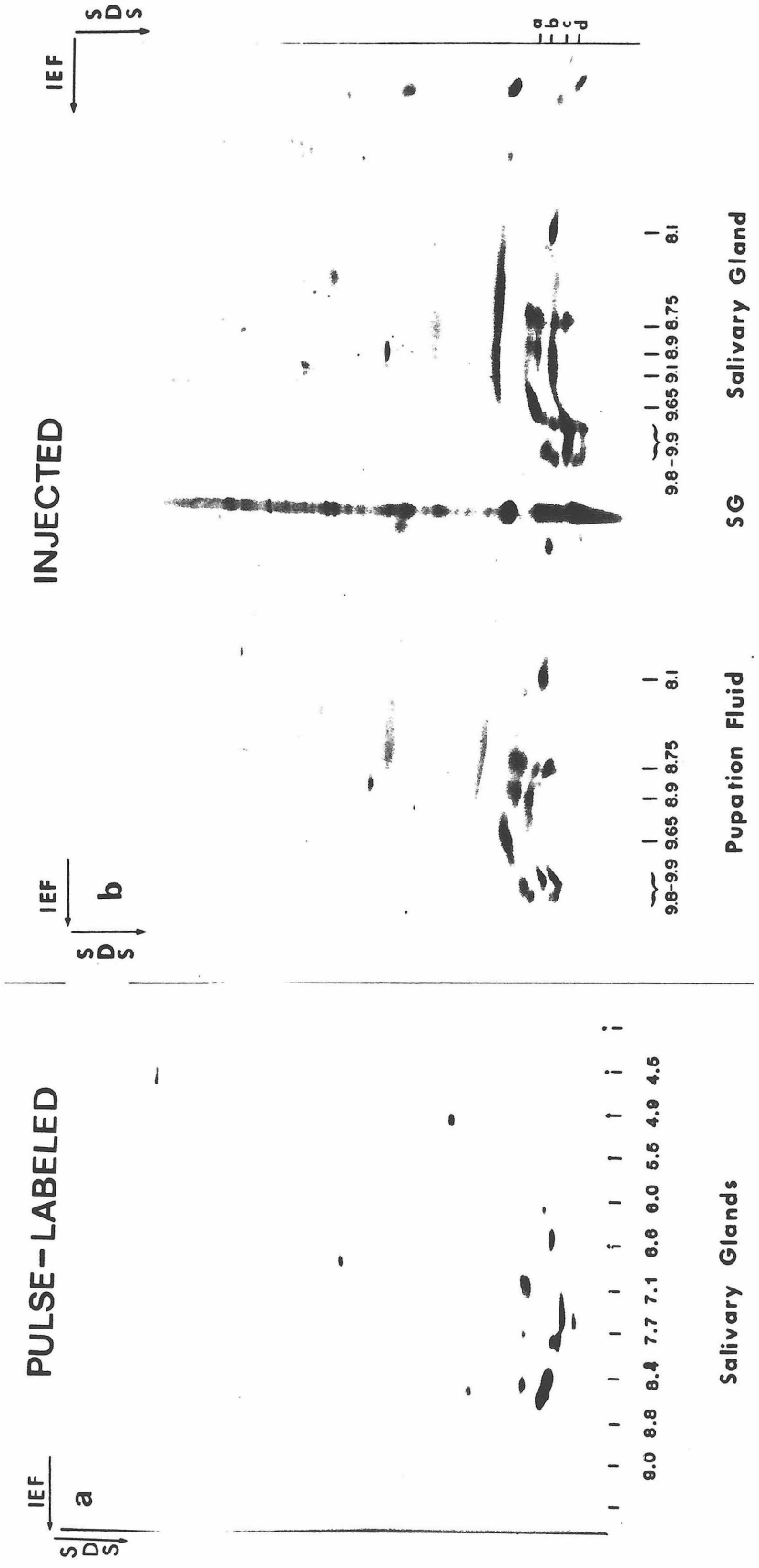

PULSE-LABELED
INJECTED
IEF
SDS
a
b
9.0 8.8 8.4 7.7 7.1 6.6 6.0 5.5 4.9 4.5
9.8-9.9 9.65 8.9 8.75 8.1
Salivary Glands
Pupation Fluid
SG
Salivary Gland
a
b
c
d

Fig. 8. Chromatofocusing profile of pulse-labeled salivary glands and the salivary gland and pupation fluid from injected animals.

Proteins from 14 pairs of salivary glands (a) and pupation fluid from 40 pupae (b). 5 hour prepupae were injected with 0.2 mCi of ^{35}S methionine and 0.2 mCi of ^{35}S cysteine in 15 µl of MOPS. Salivary glands and pupation fluid were collected at the time of pupation and the samples were processed as described in Materials and Methods. The matrix of the chromatofocusing column was an ion exchanger "PBE 94" which had been equilibrated with 0.025M ethanolamine-HCl, pH 9.4. The sample was eluted with Pharmalyte 8-10.5 adjusted to pH 8.0 with HCl. 100 µl fractions were collected and the pH and radioactivities were determined. (c) Chromatofocusing profile of proteins from 6 pairs of salivary glands from 7.0 hour prepupae which were pulse-labeled with ^{35}S methionine.

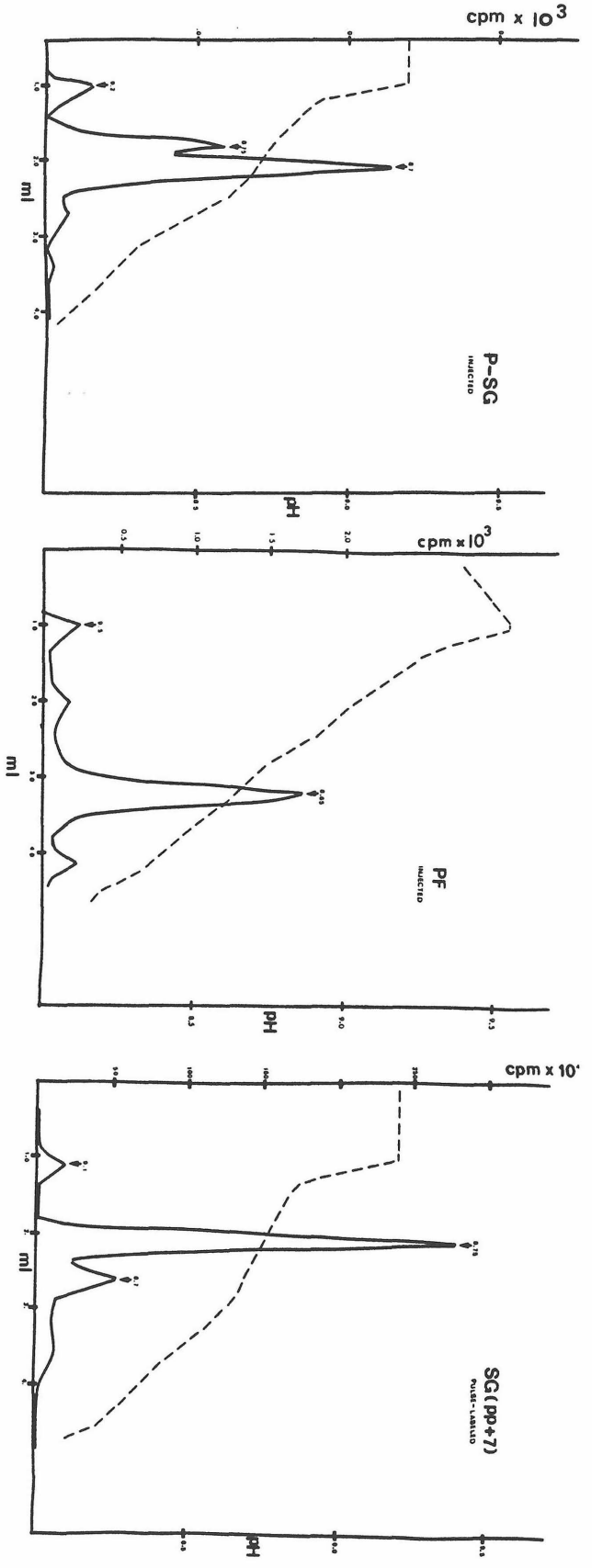
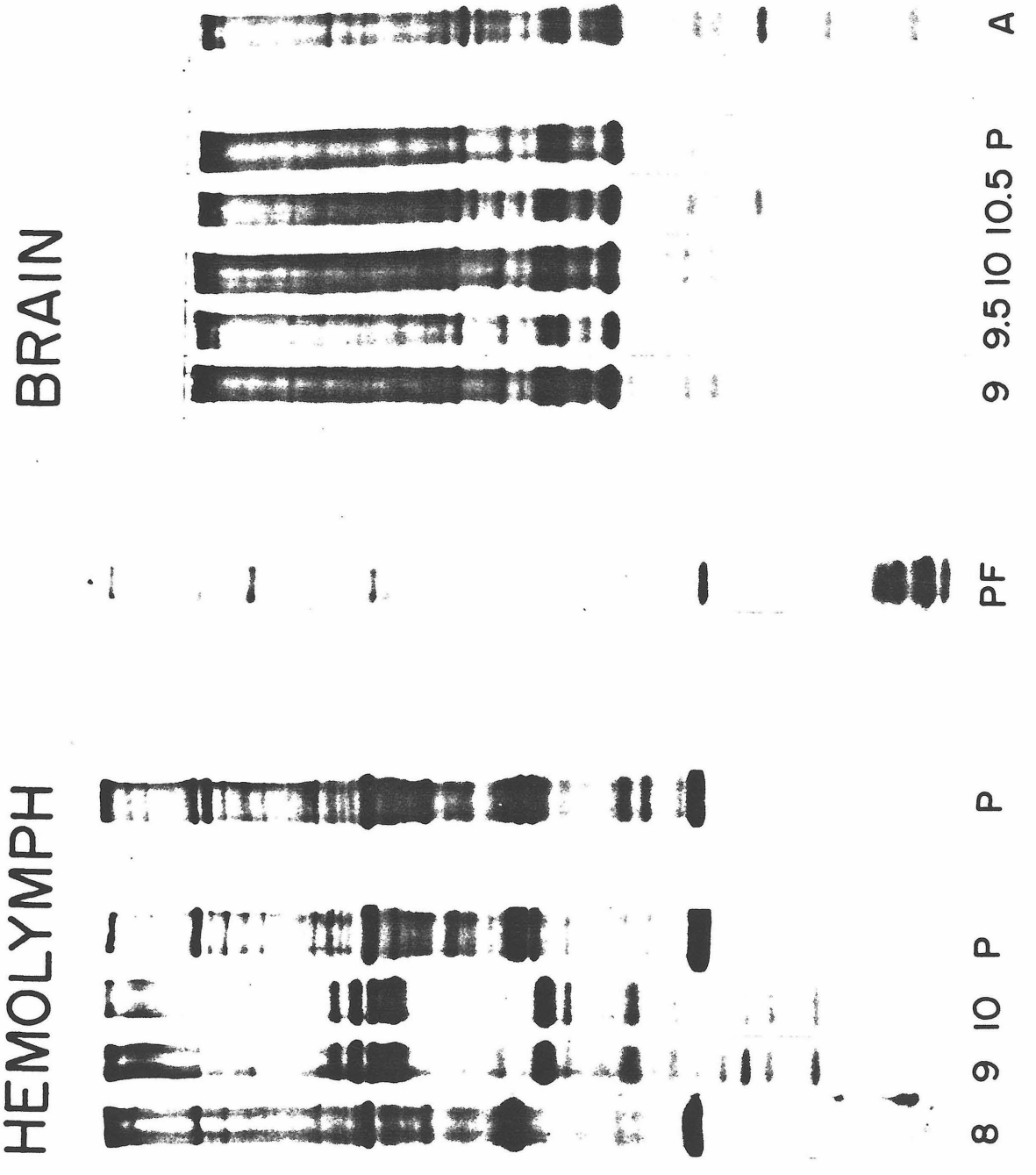

cpm x 10³
P-SG
INJECTED
pH
ml
cpm x 10³
PF
INJECTED
pH
ml
cpm x 10⁴
SG(pp+7)
POLIO-LABELED
pH
ml

Fig. 9. Autoradiograph of protein patterns from the hemolymph and brain of prepupae injected with ^{35}S methionine.

Numbers below each lane indicate the number of hours after puparium formation when the samples were taken. P refers to the sample taken when pupation occurred; PF refers to the pupation fluid collected at pupation and A is the protein pattern obtained from a whole animal when pupation occurs.


BRAIN
A
9 9.5 10 10.5 P
PF
HEMOLYMPH
P
8 9 10 P

Chapter 2

Heat Shock and Phenocopy Induction in Drosophila

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Heat Shock and Phenocopy Induction in Drosophila

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Summary

Differentiation of the single cells that yield bristles and hairs in Drosophila has been examined by studies of phenocopies induced by heat shock. Phenocopies that resemble the mutants hook and javelin are produced by application of the stress within narrow and specific time ranges during metamorphosis. Double phenocopies can be induced by successive heat shocks at appropriate time intervals. Phenocopy production may be related to heat shock effects on RNA synthesis through a feedback inhibition of transcription by heat shock proteins.

Introduction

Goldschmidt (1935) coined the term "phenocopy" to describe mutant-like morphological alterations of Drosophila that could be induced by imposition of stress during development. A stress such as heat shock was shown to yield specific phenocopies when applied at specific stages of embryological development or metamorphosis. Subsequently, Gloor (1947) with Hadorn (1955) described bithorax phenocopies resulting from treatment of 4 hr embryos by heat shock or ether, and a cryptocephal phenocopy induced by heat shock a few hours prior to head eversion. Milkman (1963, 1966) made an extensive study of wing-vein abnormalities as induced by heat shock and considered the problem in terms of possible conformational changes in specific proteins. Even now, however, no specific information as to which proteins are involved is available.

Although physical stresses such as heat shock are advantageous for phenocopy production since they can be applied and withdrawn at will, it would be remiss not to mention, and consider in terms of mechanisms, the extensive research into the production of phenocopies by chemical treatments. Rapoport (1939), Sang and McDonald (1954), and Goldschmidt and Peternick (1957) described many phenocopies in Drosophila that result from feeding with borates and other salts. Tetraborate fed to females after fertilization had drastic effects on embryos and yielded flies resembling one or more of the mutants eyeless, aristopedia, Bar, dachs, beaded and many others. At approximately the same time, Schultz, Rothman and Aronsen (1955) suggested the use of phenocopy production for screening compounds suspect in carcinogenesis. Tests that involved feeding some 30 different toxic organic compounds yielded a variety of phenocopies with high incidence. As with borate, the methods used did not permit a recognition of specific sensitive periods for production of particular phenocopies. Gehring (1964) and Rizki and Rizki (1965) subsequently produced phenocopies in Drosophila by feeding with purine and pyrimidine analogs, and Fristrom (1965) made an extensive study of the cryptocephal phenocopy produced by feeding large amounts of glucosamine. More recently, Capdevila and Garcia-Bellido (1974) repeated and extended the early work of Gloor (1947) on production of bithorax phenocopies by treatment of embryos with ether.

The investigations described in this paper are directed toward gaining a better understanding of the molecular basis of the phenocopy phenomenon and its relation to the control of development. Earlier work in this laboratory (Mitchell, 1966) demonstrated that the single gene mutant straw of Drosophila and its phenocopy (from heat shock) are both deficient in an activating enzyme (Seybold, Meltzer and Mitchell, 1975) that functions in the production of phenol oxidase. It is this enzyme (phenol oxidase) that is required for the melanin production that yields the normal, nearly black bristles of the adult fly. Both straw and its phenocopy have blond bristles on the head and thorax. Thus the same molecular system appeared to be affected in the mutant and the phenocopy. In this particular case of phenocopy production, the evidence suggested a heat shock effect directly on transcription of a gene that must be expressed in a specific time interval to result in normal development. A model for such a program of gene expression was subsequently developed (Tissières, Mitchell and Tracy, 1974) through studies of heat shock effects on protein synthesis in salivary glands and other tissues of Drosophila. It was demonstrated that heat shock induces synthesis of specific new proteins and causes cessation of much of the protein synthesis in progress. These specific changes correlated very well with changes in puff patterns in salivary gland chromosomes as described by Ashburner (1967). Since transcriptional activity is part of the puffing process that can be observed in salivary gland chromosomes, an important result of heat shock is extensive modification of transcriptional activities. Extensive additional investigations in several laboratories (McKenzie, Henikoff and Meselson, 1975; Ish-Horowicz, Holden and Gehring, 1977; Spradling, Pardue and Penman, Mirault et al., 1978; Moran et al., 1978) have demonstrated conclusively that the induced protein synthesis (from heat shock) is the

result of induction of transcription at specific sites on the chromosomes. The cessation of synthesis in progress at the time of heat shock is probably due to cessation of transcriptional activity and inactivation of existing message. This supposition remains to be confirmed.

For the investigation described here, we chose to study a period in the pupal development of Drosophila during which the lethal effects of heat shock are minimal (Mitchell, 1966) but the need for structural proteins is high. We have assumed that the synthesis of relatively large quantities of structural proteins (and mRNAs) will facilitate comparisons of the primary molecules involved in the production of a mutant and its phenocopy. For this reason, we have looked at effects on bristles and hairs. Each of these is formed from within cytoplasmic extrusions of hypodermal cells (Bodenstein, 1950). It is not known how the construction is regulated or whether it starts from the tip, the base or elsewhere. The general form is evident by 55 hr after puparium formation as observed with the scanning electron microscope (Mitchell and Lipps, 1977).

Results

Phenocopy Frequency

The information summarized in Figure 1 was derived from examination of emerged adults 3–4 days after heat shock treatment at 40.2°C for 40 min. In this age range (30–50 hr after puparium formation), 30–50% of the animals emerge under these conditions and virtually all the animals continue development to the time at which emergence should occur. Those that do not eclose when removed from the case show the observable phenocopies to a greater extent than the emerged flies. That is, for some of the emerged flies, only one scutellar was bristle-affected, whereas for noneclosed animals, all four were affected and the complexity of the structure was usually greater.

Figure 1 shows data for four separate phenocopies designated as angle bristle, smooth bristle, multiple hairs and spear bristle. Each experimental point was derived from a heat treatment of at least 100 pupae at the average age (post-puparium) indicated. As shown, approximately 90% of the emerged flies demonstrate the character indicated near the time of maximum sensitivity. These are minimum values, since all nonemerged animals show the characters in question. These data demonstrate clearly that the sensitive periods for phenocopy production are quite short—probably less than 2 hr, since the animals were originally synchronized to only approximately ± 1 hr. It should also be noted in this connection that all the data plotted in Figure 1 came from the same experiment

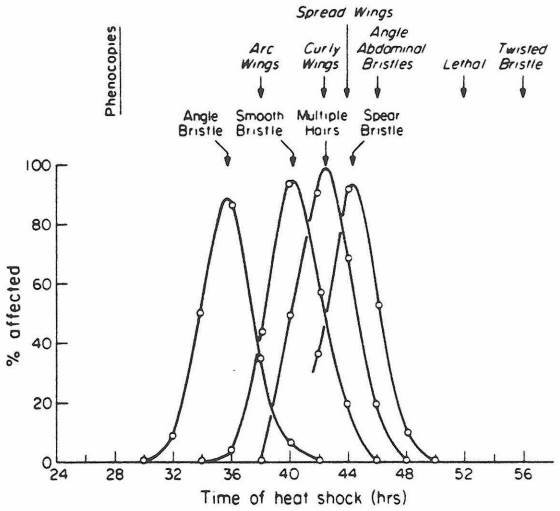

Figure 1. Phenocopy Frequencies (as Percentage of Emerged Flies)

Pupae synchronized within ± 1 hr were heat-shocked at 40.2°C for 40 min at the ages indicated (timed from puparium formation). They were then incubated at 25°C until emergence 3–4 days later and scored for phenotypes. Data are given for the four characters—angle bristles, smooth bristles, multiple hairs and spear bristles. The six characters shown at the top were scored but are not considered further here.

More detailed descriptions of the four phenotypes are given in Figures 3–7.

Sensitive periods are short (<5 hr), but more than 90% of emerged flies are affected.

with one set of synchronized animals. In numerous other experiments with other sets of animals, we have observed that while the time scale may be shifted as much as 2 hr, the relative positions of sensitivity maxima are always the same. Although angle bristles and smooth bristles, for example, appear a little over 4 hr apart, the maximum for angle bristles may fall between 34 and 38 hr.

Relative positions on the time scale for six other phenotype characters are also indicated in Figure 1. The three wing characters are designated by the names of the mutants which they resemble (Lindsley and Grell, 1968); the lethal and twisted (minute) bristle characters were described previously (Mitchell, 1966). Although a variety of other characters have been noted for this 20 hr period, details have not yet been determined.

Morphology

To provide a basis for comparison of mutants and phenocopies, some characteristics of normal scutellar bristles are shown in Figure 2. This very regular structure, which is derived from a single cell, is shown here in seven sections. The fluting that extends from socket to tip has similar dimensions throughout, and thus the tapering and slight overall curvature is determined by reductions in flute numbers (as in the fourth section from the left

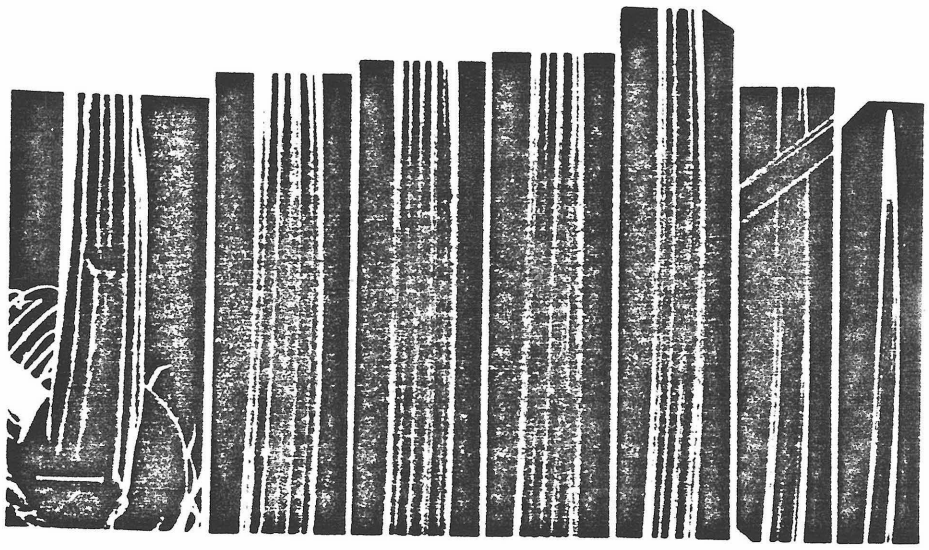

Figure 2. Normal Scutellar Bristle

A complete scutellar bristle from a wild-type fly is shown beginning with the socket at the left to the tip at the right. The photograph was taken with the scanning electron microscope. Bar = 5 μ.

The bristle has a regular taper (and slight curvature) from base to tip and is fluted from base to tip.

The fluting is regular and of similar dimensions so that the taper and curvature are determined primarily by an orderly reduction in the number of flutes. An example of flute reduction is shown in the fourth segment from the left.

in Figure 2) at appropriate intervals. A molecular basis for this structural determination may be arrived at by means of some of the phenocopies and mutants described as follows.

The characteristics of phenocopies described in Figure 1 are illustrated in Figures 3-7. In Figure 3, two sections each of a scutellar bristle from an emerged fly are shown. The numbers refer to the time scale in Figure 1—that is, the photograph labeled 30 shows the base and tip of a bristle from an animal that was heat-shocked at 30 hr after puparium formation (approximately 3 days before emergence). The intervening sections (4-5 in number as in Figure 2) that are not shown are fluted or smooth like the two parts that are shown. Thus the 30 hr sample is essentially normal. The 36 hr sample shows fluting at the base with some smoothness in this particular example, but more significantly, it shows the angle bristle character (Figure 3, upper panel). The rest of the bristle both proximal and distal to the angle is fluted, and in many cases the base is completely normal. At 40 hr, the bristle is smooth throughout its length. It is unevenly tapered; often it is flat and ribbon-like toward the tip and often it has an open end (Figure 3, 40 hr). Although the example presented for the spear bristle (44 hr) is typical, the spear tip varies considerably in form. The entire bristle is fluted, but in a rather irregular fashion as shown in the base section. Finally, in this series, the 50 hr sample approaches the normal structure except that the flutes may be larger in size and fewer in number. This structure may demonstrate an additional phenocopy type, although this possibility has not yet been examined in detail. We know from earlier studies (Mitchell, 1966) that there are at least two and perhaps several more phenocopies which can be induced in these bristles in the next 20 hr. After that time, heat shock has no effect on the forms of scutellar bristles.

Unlike bristles, which include a second cell as a socket, hairs are single protrusions from single cells. Normal hairs of the scutellum and thorax are slightly fluted, as shown in the upper left-hand panel of Figure 4. The upper right-hand panel shows a similar area from a 42 hr heat-shocked animal, and the lower panels show higher magnifications of some of the phenocopy (multiple hairs) variations in form. Although it has not been determined by sectioning, these variations appear to be multiple protrusions from single cells. The hairs do tend to be lacking in flutes and many have holes in the ends. At the most sensitive period (42 hr), all hairs on the dorsal thorax and scutellum are affected; at 40 and 44 hr, however, some are normal. For the data in Figure 4, the effect was considered positive when even a small proportion (approximately 10%) of the thoracic hairs were abnormal

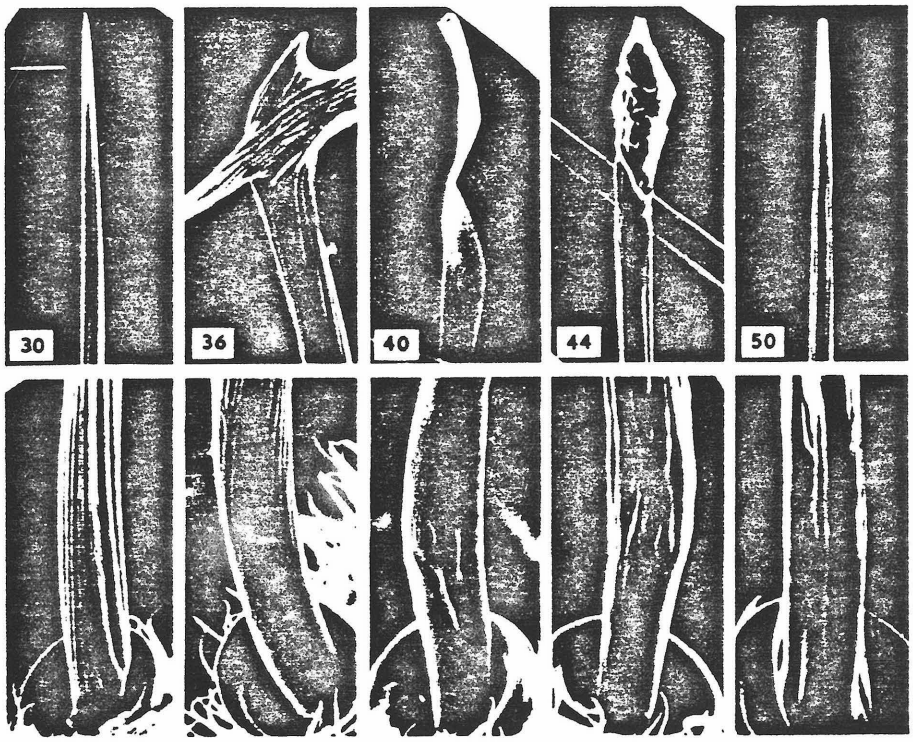

Figure 3. Bristle Phenocopies (See Figure 1)

Scanning electron microscope photographs of scutellar bristles corresponding to the data in Figure 1. Each photograph shows the base of the bristle and another significant portion. The segments not shown are like those shown—for example, the smooth bristle from 40 hr is smooth throughout its length. Bar = 5 μ.

The sample from 30 hr heating is essentially normal and that from 50 hr is nearly so.

The three bristle phenotypes from 36, 40 and 44 hr (see Figure 1) are easily distinguishable. There are variations in form and other examples are shown in Figures 4–6. The tips of the 36 hr bristles are normal.

Phenocopies and Mutants

The phenocopy character that is described in Figures 1 and 3 (36 hr) bears a close resemblance to the phenotype of the mutant hook. The mutant *hk* is shown in the upper row of photographs in Figure 5 and the phenocopy (PhC) in the lower row. In both cases, the lower magnification photographs show all four scutellar bristles and all four are affected. This is not always true in either the phenocopy or the mutant; it is true that, in general, the phenocopy shows the more extreme abnormality. The phenocopy also tends to show more extreme and bizarre rearrangements at the point of bending of the bristles. Some variations of this characteristic are shown in the higher magnification photographs in Figure 5.

Although the similarities between the mutant *hk* and the phenocopy (angle bristle) are remarkable, the differences are also of interest. In the phenocopy it is unusual to find any bristles, except for the scutellars, affected by the heat shock at 36 hr. As shown in Figure 6, however, angle bristles can be induced on the abdomen at 46 hr. At this time, the scutellars are not affected in this way. In a similar fashion, the large stemopleural bristles become hooked as a result of a heat shock at 42 hr but not before or after.

In contrast, the mutant *hk* shows the mutant phenotype on the abdomen and the head as well as on the scutellum. The frequency is low for the abdomen, but nearly every fly has a few angled bristles in this area. These observations emphasize the fact that all cells in *hk* carry the mutant gene and that it is expressed when appropriate. On the other hand, the phenocopy represents effects of stress at different points in time, and if it can be shown that the stress affects the same gene products that are altered in the mutant, then the phenocopy can serve very well to define developmental stages at which the gene involved is expressed.

The cases of smooth bristles, spear bristles and the mutant javelin (*jv*) are considered together for the reasons summarized in Figure 7. Two complete scutellar bristles from *jv* and a base and tip are displayed in the center of the figure. Each has a smooth base which extends about halfway to the tip. From this point on, fluting appears and extends to the tip even through the various spear-like ends.

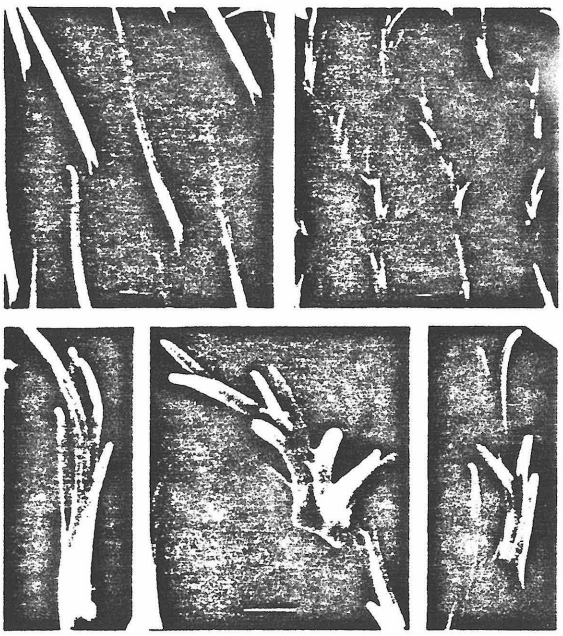

Figure 4. Multiple-Hair Phenocopy

An area of the dorsal thorax showing normal hairs is shown in the upper left-hand panel, and a similar area from heat shock phenocopy is shown in the upper right-hand panel. Higher magnifications of the abnormal hairs are shown in the three lower panels. Bars = 1 μ.

The *jv* phenotype is therefore copied in the proximal half by the smooth bristle phenocopy (left in Figure 7; see also Figures 1 and 3) and in the distal half by the spear bristle phenocopy (right in Figure 7). Again as in the previous case involving the mutant *hk*, the phenocopies appear to be more restricted in areas of expression and more specific in morphological effects than the mutant.

Regarding the fourth phenocopy character (Figure 4) (that of multiple hairs), we have not yet found a mutant of which it is a copy. The mutant multiple wing hairs (*mwh*) is expressed on the thorax as well as the wings, but it frequently has four or five hairs coming from a single cell. This seems to be the case in the phenocopy (Figure 4), but in the mutant, the hairs are spaced separately; they are not in bundles nor are they branched. It is possible, of course, that the phenocopy is simply more extreme than this mutant. The phenocopy is not expressed on the wings with the 42 hr heat shock, but we have not looked extensively for sensitive periods affecting hairs on areas other than the thorax.

Double Phenocopies

The data in Figure 1 and earlier observations (Mitchell, 1966) indicate the involvement of independent events in the production of the different kinds of phenocopies described. This presumption is supported further by the information summarized in Table 1 and Figure 8. In this experiment, double and triple heat shocks were applied as shown in the first column of Table 1. A temperature of 40°C rather than 40.2°C was used to reduce lethality and also to allow an additional means of evaluating the double heat shock effects—that is, at 40°C only about two thirds of the animals show the angle or *hk* phenocopy (Table 1). The results from a second heat sock can then be more easily observed as an enhancement of the first effect or as a superimposition of a second effect. This temperature difference is also significant in relation to the heat-induced delay in development described earlier (Mitchell, 1966), in Figure 10 and elsewhere by Lindsley and Poodry (1977). This phenomenon is evident in Table 1, which shows that a single heat shock yielded 61% *hk* phenocopies, which second and third shocks with 4 and 5 hr intervals, respectively, yielded only more of the same phenotype even though the time span covered the sensitive periods for both smooth and spear. On the other hand, the second shock after a 9 hr interval and the third at 9 plus 5 did not enhance angle (*hk*), but did yield doubles of angle-smooth in many animals. Doubles of angle-spear were obtained from treatments of animals using a 15 hr interval between the first and second shocks. Data on the multiple hair phenocopy are not included here since different cells are involved, but this character was present in all cases where smooth was observed.

Figure 8 shows some examples of the morphology of bristles observed in this experiment (summarized in Table 1). Figure 8A (from a single heat shock) shows a more or less typical angle which is about halfway between a simple bend and complex bend such as that shown in Figure 8B. The latter is from the triple shock (36, 40, 45), and overall these are more extreme than the products from single shocks. The bristle in Figure 8C is from the triple shock 36, 45, 50 and shows a fairly extreme angle plus a mild smooth. This time sequence apparently carried the animals a little past the peak for smooth. Even though the percentage was high, smooth effects were less extreme than with the double shock, and a significant yield of spear was obtained in the triple treatment. Figure 8D is from the double heat shock with a 15 hr interval, and it shows angle as a simple bend and a spear tip.

These examples (Table 1 and Figure 8) are all concerned with double phenocopies and not with mutants. It is of interest to note, however, that when the mutant *hk* was subjected to heat shock at the angle phenocopy-sensitive period, the hook character became more extreme both in the numbers of bristles affected and in the complexity of the bristle structure.

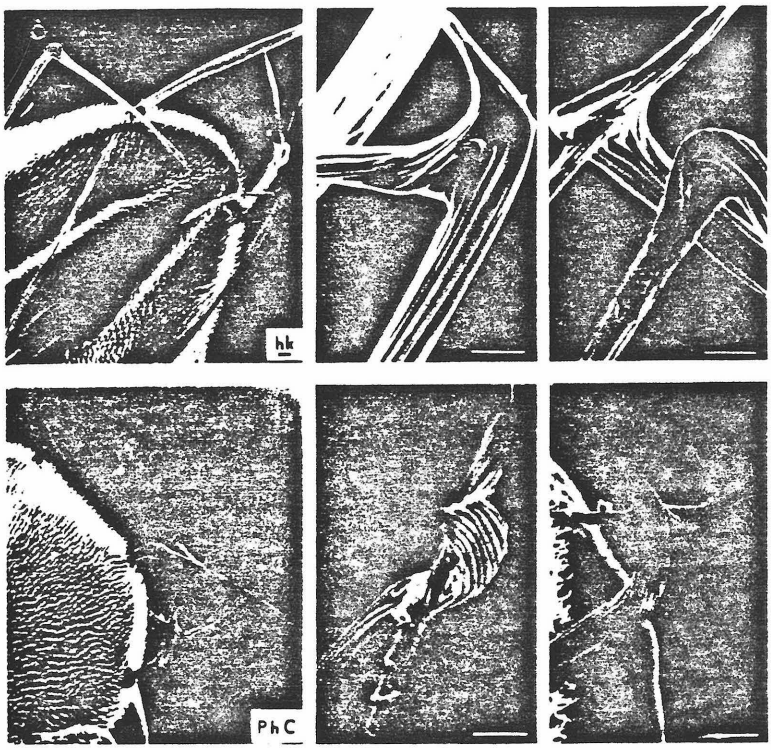

Figure 5. Comparison of the Mutant hook with the Angle Bristle (36 Hr) Phenocopy

The scanning electron microscope photographs at the left show the scutellar bristles of *hk* and the angle bristle phenocopy (PhC). Higher magnifications (bars = 5 μ) are shown at the right for each case.

The angular bends in the bristles take various forms both as simple bends and as complex structures. There is a complete overlap in the various forms taken in the mutant and the phenocopy.

The cell hairs shown in the background at the left are essentially normal.

Protein Synthesis

The phenocopies described in both bristles and hairs must result from heat shock effects on the single cells from which these structures are formed. We have previously studied the changing patterns of protein synthesis in these cells during normal development. (Mitchell, Lipps and Tracy, 1977). This broad picture is supplemented in more detail in Figure 9, which shows normal patterns of pulse labeling in the pupal period of 32–50 hr. The figure is from a direct autoradiograph of an SDS gel. The animals were of the mutant *jv*, and the labeling was by incubation (30 min) of the dorsal thoracic hypoderm from three animals in buffered ^{35}S-methionine. Virtually identical results have been obtained with two wild strains (Canton S and Urbana S). Figure 9, from *jv* animals, is used here only because it had the complete time sequence on the same gel. In the present context, the salient observations are the following:

—Of about 30 bands which show in each track, at least 24 appear and/or disappear during the time interval from 32–50 hr. Some of the changing components are marked at the left of Figure 9.

—These rapidly changing patterns are much like those observed in salivary glands (Tissières et al., 1974) and must be related to changing cell functions during development.

Heat Shock, Transcription and Translation

Earlier work on the production of phenol oxidase in the mutant straw and its phenocopy (Mitchell, 1966; Seybold et al., 1975) suggested that RNA and/or protein synthesis is subdued for a period of time after heat shock, followed by a period of recovery. This is indeed the result obtained. Figure 10 shows patterns of synthesis of RNA and proteins obtained from an experiment with one set of synchronized animals (Canton S) at the 34 hr stage in pupal development. The animals were heat-shocked at 40.2°C for 40 min, and samples were taken for dissection and labeling with ^{3}H-nucleosides or ^{35}S-methionine at the intervals (hr) indicated. Shocked animals not used were retained at 25°C until the flies emerged, and these showed 62% angle (*hk*) phenotype (see Figures 1, 3 and 5).

The important matter to consider in this experiment is the comparison of heat shock effects on RNA and protein synthesis. RNA patterns, from fluorography of a formamide gel (Mitchell et al.

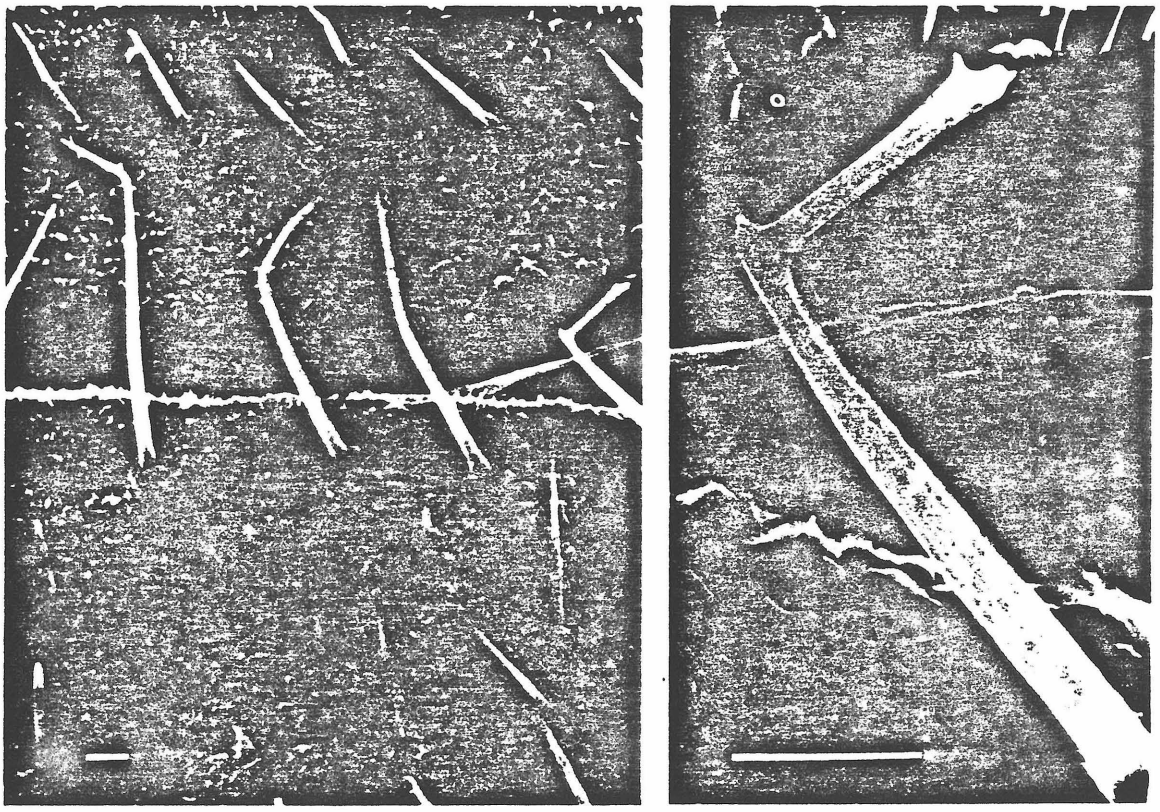

Figure 6. Angle Bristles (*hk* Types) on the Abdomen

The bristles shown are from a scanning electron microscope photograph of the fourth abdominal segment of a fly that had been given a heat shock (at 40.2°C for 40 min) at 46 hr in the pupal stage. Bar = 5 μ.

These angle bristles on the abdomen resulted from a heat shock at 46 hr; none was observed at 36 hr when the scutellars are affected. The angle or *hk* bristle type was not produced in the scutellars at 46 hr.

The mutant *hk* has strongly affected scutellar bristles (Figure 4), but nearly all adult flies also have at least one angle-type abdominal bristle.

1978), are shown at the top of Figure 10 with the nonheated control at the left. This typical pattern for denatured RNA from whole tissue was visible for the control as well as the 11, 16 and 23 hr samples after a 5 day exposure of the film to the gel. The photograph presented here is from an exposure of 5 months. Thus, at the minimum RNA level (2 hr after the heat shock), transcriptional activity is reduced to much less than 1% of the normal level. On the other hand, the reduction in the translation level (Figure 10, lower panel) is estimated to be more in the range of 1–10% of normal.

Another matter of considerable interest shown in Figure 10 is the range in which heat shock proteins are present in relation to total RNA synthesis. As shown in the lower panel, the heat shock proteins are strong by 4 hr after the shock and their synthesis ceases in the range of 7–11 hr. During this period, recovery of a more normal pattern of protein synthesis is also evident, and this coincides with the resumption of general RNA synthesis. This latter is in contrast to the 1–4 hr period when RNA synthesis is minimal but the production of heat shock message is necessarily at a maximum. These results therefore suggest that a matter for consideration in phenocopy production concerns the nature of priorities in transcriptional activities.

Discussion

The Phenocopies

The experimental work described here presents the production of a specific phenocopy in Drosophila as a single event directly related to the programming of development. Each of the phenocopies concerned with the struture of a bristle or hair is induced in the given cell type at a specific time but only when the cell is in a susceptible state. A clear example is that of the phenocopy angle, where the mutant copy can be induced at 36 hr in the cells that become scutellar bristles, at 42 hr in the cells that become sternopleural bristles, and at 46 hr in cells that yield abdominal bristles. This dissection

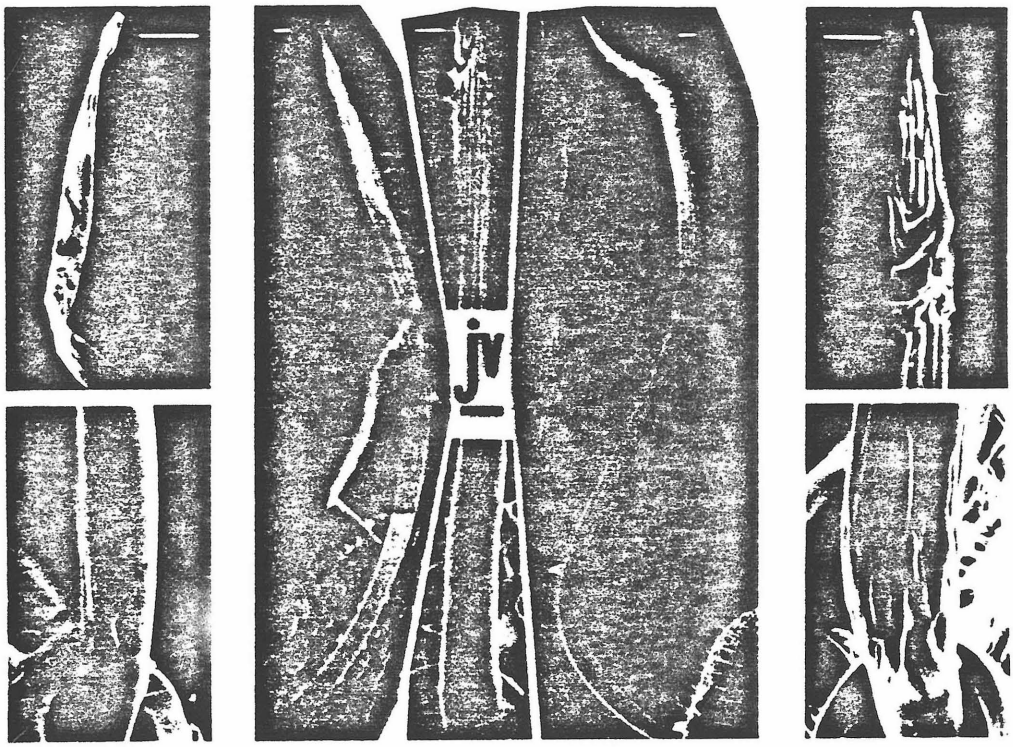

Figure 7. The Mutant javelin and the Phenocopies Smooth and Spear

Three examples of scutellar bristles from the mutant *jv* are shown in the center of the figure. The base and tip of a smooth type phenocopy are shown at the left, and a spear type is shown at the right. Bar = 5 μ.

The mutant *jv* has a smooth base, a fluted distal portion and a spear tip. The spear tip has various but similar forms in both the mutant and the phenocopy.

The *jv* mutant affects bristles other than the scutellars, but no information on phenocopy periods other than that given in Figure 1 is yet available.

Table 1. Phenocopies from Double and Triple Heat Shocks

Heat Shocks[a] (Hr)	Emerged Flies (%)	Emergence Delay (Hr)	Phenocopies (%)		
			Angle (*hk*)	Smooth	Spear
None	96	0	0	0	0
36	85	4	61	0	0
36, 40	80	5.5	100	0	0
36, 40, 45	44	8.5	100	0	0
36, 45	78	5	61	48	0
36, 46, 50	69	8.5	66	82	6
36, 51	75	6.5	62	0	70

[a] Heat shocks were 40°C for 40 min starting with 36 hr pupae. Second and third shocks were given at the hours indicated with incubations before and after at 25°C.

of a phenotype based on time is in contrast to the effects of the mutation in *hk*. Since the mutant gene is always present, it is expressed whenever a cell has reached the appropriate stage. The situation with the mutant *jv* and the semi-copies smooth and spear could be explained on a similar basis—that is, the *jv* phenotype could come from a double expression of the same gene 4 hr apart with the same gene product providing for smooth the first time and spear the second. There is no direct evidence at present to support this explanation, but double expressions are frequent where they

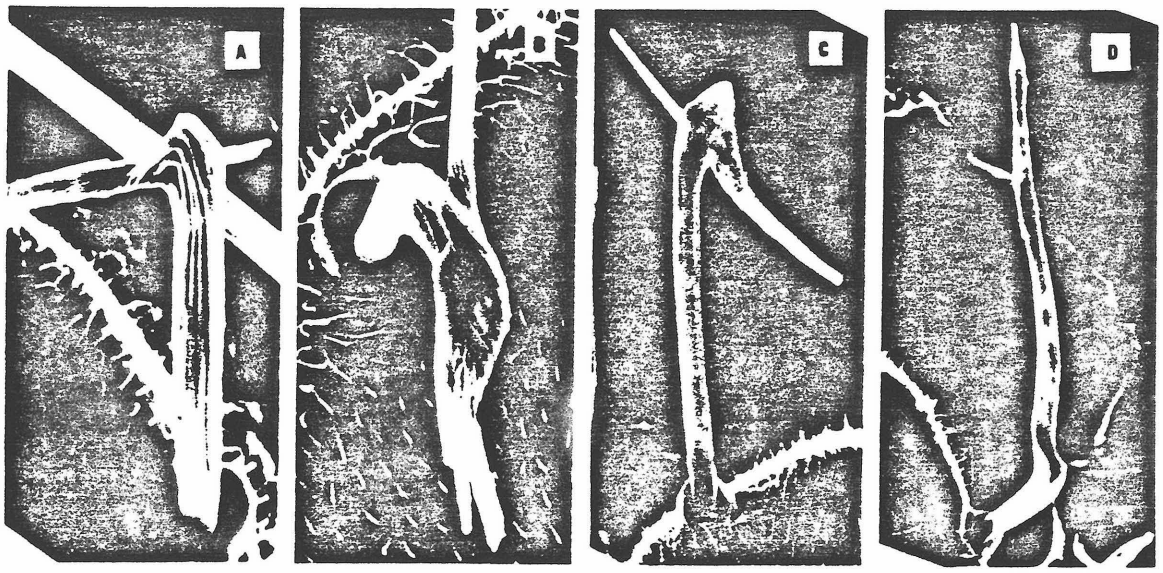

Figure 8. Double Phenocopies

The examples shown are from the experiment summarized in Table 1. (A) is from a single heat shock showing an angle-type bend of medium complexity. (B) is from the triple heat shock (36, 40, 45) and shows a bend of higher complexity but still not smooth or spear. (C) shows a double phenocopy of angle and smooth from the 36, 45, 50 treament. (D) is from the 36, 51 double heat shock and shows a double phenocopy of a simple bend (angle) and spear. Bars = 10 μ.

are easily observed as chromosome puffs in the salivary glands (Ashburner, 1967). Such rapid changes in patterns of gene products (as proteins) also occur in epithelial tissue as shown earlier (Mitchell and Lipps, 1977) and in Figure 9.

It should be pointed out that the choice was made for this investigation of structural changes in bristles and hairs with the expectation that one or more of the mutants and phenocopies would have a deficiency or modification of a structural protein such as actin or tubulin. No evidence is yet available on this point, and we may be dealing with a catalytic component as in the case examined earlier (Mitchell, 1966; Seybold et al., 1975).

Potential Mechanisms

It was postulated earlier (Mitchell, 1966) that heat shock induces phenocopies by interfering with the process of primary gene activity—that is, transcription. This remains a reasonable general postulation, but there are now more details to be considered. The following facts are pertinent:

—Heat shock causes induction of transcriptional activity at a small number of specific chromosomal loci.

—Heat shock results in an extreme reduction of RNA synthesis in general and in regression of transcription at chromosome loci which are active at the time of the shock.

—Following heat shock, development is suspended for several hours along with reduced transcriptional activity.

—Heat shock proteins derived from the heat-induced mRNAs are synthesized during the suspended state, and most of them concentrate in the cell nucleus.

—The suspended state can be prolonged by a second and third heat shock if the stress is reapplied while the heat shock proteins are still in production.

—Cessation of synthesis of the heat shock proteins is coincident with the restoration of general RNA synthesis.

—As RNA synthesis is restored, so is a pattern of protein synthesis similar to that existing before application of the heat shock.

—Neither regression nor restoration of protein synthesis patterns is coordinate.

The first two facts in this list are now very well documented (Tissières et al., 1974; McKenzie et al., 1975; Ish-Horowicz et al., 1977; Spradling et al., 1977; Mirault et al., 1978; Moran et al., 1978). The third point on developmental delay was described earlier (Mitchell, 1966; Lindsley and Poodry, 1977) and briefly in Figure 10. A much more extensive description of the phenomenon in terms of protein synthesis patterns in different tissues is in preparation in this laboratory (by A. Chomyn, G. Moller and H. K. Mitchell).

The fourth point mentioned above may be the most critical of all. We demonstrated earlier (Mitchell and Lipps, 1975) that the heat shock proteins

Figure 9. Pulse-Label Time Series

Pupae from the mutant javelin were dissected at the ages indicated, and the dorsal epithelium of the thorax was incubated for 30 min (at 25°C) with ^{35}S-methionine. Labeled proteins were separated by electrophoresis on SDS-acrylamide gels and then detected by contact autoradiography. The sample at the far right (44H) was identical to the 44 hr sample, except that the pupae were heated at 40.2°C for 40 min and then kept at 25°C for 8 hr before dissection. Two of the proteins induced by heat shock are marked as 70 K and 22 K (representing molecular weights of 70,000 and 22,000 daltons). The other prominent heat shock proteins are at 84, 68, 34, 27, 26 and 23 K.

Over this time period of pupal development, the synthesis of most of the pulse-labeled polypeptides was turned on, turned off, or both on and then off. In the equivalent situation in salivary glands where chromosomes can be observed directly, such pattern changes have been shown to result from controls at the transcriptional level.

(except for the 84 k component) return rapidly after synthesis to the nuclei of salivary glands where they evidently replace the normal complement of nonhistone chromosomal proteins. It may be a coincidence, but as shown in Figure 10, the state of suspended development as well as the suppression of general RNA synthesis is retained until the synthesis of heat shock protein ceases.

From a consideration of these various facets of the system and the effects of heat shock upon it, the following sequence of events could account for phenocopy induction:

—Heat shock causes a dissociation of a common repressor at a set of susceptible genetic loci.

—mRNAs are produced at these loci, and they are exported to the cytoplasm and translated.

—The heat shock proteins return to the nucleus and act as general repressors of existing RNA synthesis at all loci except those from which their

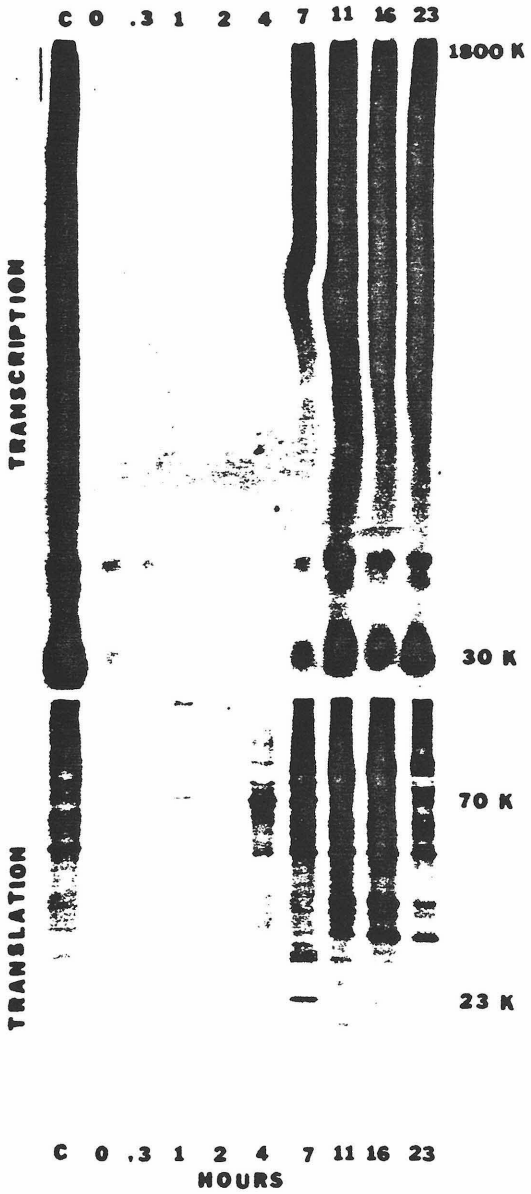

Figure 10. Heat Shock Effects on Transcription and Translation

The upper panel shows the results of pulse labeling of thoracic hypoderm with ^{3}H-nucleosides to show total RNA. A nonheated control is shown at the left (C), and the numbers on the other tracks are time (hr) at 25°C after completion of the heat shock (at 40.2°C for 40 min). An approximate molecular weight scale is shown at the right. Markers used (1800 K and 30 K) were from a commercial preparation of rRNA which also contained 4S RNA. Neither the visible bands nor others (see Mitchell et al., 1978) obscured by the smear of heterogeneous material have yet been identified. (Fluorograph; formamide gel; exposure time 5 months at −80°C.)

The lower panel shows results from pulse labeling with ^{35}S-methionine. The experiments on RNA and protein synthesis were performed at the same time with the same set of synchronized animals. The protein patterns are from a direct autoradiograph with an exposure time of 79 hr. Note the heat shock proteins in the 4 and 7 hr samples (70 K and 23 K components are marked), all of which are no longer made by 11 hr. Note also that heat shock messengers must be present at 4 hr, but they are not in a high enough concentration to show in the 4 hr RNA pattern.

own messages originated.

– The recovery process after heat shock is differential at different chromosomal loci.

– A phenocopy originates from a lack of recovery of function in time to participate in continuing development.

In this model, the transitory repression of a transcriptional activity is equivalent to a lack of fuction or an abnormal function in a mutant, time of phenocopy production corresponds to the time of expression of the normal allele of the mutant gene. Obviously, there are many variations possible in such a scheme, and a critical step can occur in a conformational change in a protein as proposed by Milkman (1963, 1966) many years ago, or it can occur at a translational level. In any case, means are now available for a further evaluation of the problem.

Experimental Procedures

Fly Stocks

Oregon R wild stock was used for most of the phenocopy preparations, as well as Canton S and Urbana S from the stock center at the California Institute of Technology. Urbana S is about 2 hr per day slower in its whole life cycle than Oregon R, but it reacts in the same way to heat shock. Mutants were obtained from the fly stocks kept at the Pasadena Stock Center. For obtaining large numbers of synchronized animals, we used the mass culture method described earlier (Mitchell and Mitchell, 1964). 2 hr egg collections yielded 2000–5000 larvae in a stock cage of about 10,000–25,000 flies. A slurry of fresh yeast was provided as needed and always by the third day after egg laying. On the fifth day, when approximately half the animals on the lid of the culture box had formed puparia, the animals were washed out of the lid with water (20–25°C) and those which floated were discarded. (The flotation process should take less than 5 min.) The remaining animals were spread on moist filter paper and kept at 25°C and 66% humidity for 2 hr. The flotation process was then repeated, the animals with 2 hr synchrony (5–7 hr post-pupariation) were retained and a new sample was obtained after the next 2 hr. Usually three and sometimes four successive floats yield good animals. Double floats improve synchrony, but in any case, animals were picked over under a dissecting microscope once or twice a day to remove any misshapen, damaged or abnormal individuals. Prepupae without extruded spiracles and clumps with two or more stuck together were also discarded. By these means, samples with nearly complete synchrony and better than 95% viability were easily obtained. They were kept at 25°C and high humidity until use.

Heat Treatments

Samples to be heat-shocked (usually 50–100 animals) were weighed or counted into test tubes. For heating, the tubes were placed in a water bath with the simultaneous addition of 1 ml of water equilibrated at the temperature of the bath (40.2°C unless otherwise designated). In these experiments, the bath temperature fluctuated <0.1°C; control to this level is essential for uniform results and high frequencies of phenocopies. Termination of the heat treatment was achieved by the addition of water (20–25°C) to about 10 vol. The animals were filtered off on a strainer and transferred to vials with fly food for subsequent observations.

Samples for SEM

Samples for observation by the scanning electron microscope were dissected and placed immediately on 1 cm^2 glass squares cut from a microscope slide. No glue was needed for freshly dissected samples, but a small piece of double-faced Scotch tape was used for wings and other dry samples. The glass squares were then glued to the aluminum holders (ETEC electron microscope) with metal glue and the samples were gold-coated.

Gel Electrophoresis

Epithelial tissues from the dorsal thorax of pupae at different stages of development were dissected and labeled with ^{35}S-methionine for 30 min as described previously (Mitchell and Lipps, 1977). The SDS gel used in this case was 15% acrylamide and 0.2% N,N′ methylene-bisacrylamide. Samples for RNA gels were dissected and labeled (with ^{3}H-nucleosides) in the same manner as for the SDS gels. After labeling, the tissues were ground in microgrinders in lactic-acetic-orcein (Mitchell et al., 1978), washed with ethanol and phenol-extracted, and the RNA was alcohol-precipitated. Denaturation was at 100°C in 98% formamide for 4 min. Formamide gel preparation and electrophoresis were carried out as described elsewhere (Mitchell et al., 1978).

Acknowledgments

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Chapter 3

Specific Protection from Phenocopy Induction by Heat Shock

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Specific Protection From Phenocopy Induction by Heat Shock

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Mild heat treatments applied to whole animals or cell cultures of Drosophila prior to lethal heat shocks result in increased survival and protection against phenocopy induction. The optimal condition for the preliminary mild heat treatment is that which induces the synthesis of heat-shock proteins but does not turn off the protein synthesis that is in progress. Recovery of protein synthesis but not RNA synthesis following a drastic heat shock is much enhanced by the pretreatments. The results suggest that the protection for survival and against phenocopy induction is due to storage of messenger RNA.

Key words: drosophila, gene regulation, heat shock, protection phenocopies, survival

INTRODUCTION

We recently described [1] a series of phenocopies that are induced in Drosophila melanogaster by subjecting pupa to heat shock at specific stages of development. Three of the phenocopies produced by shocks at successive intervals closely resemble the mutants hook and javelin which are involved in determining the structure of the scutellar bristles of the adult fly. We have also shown that the conditions used for phenocopy induction, turn off, for a time, both transciptional and translational activities in Drosophila tissues [1, 2]. Subsequently, translation resumes long before reactivation of transcription [1]. These findings show that heat shock can result in storage of mRNAs in keeping with the observations of McKenzie et al [13] and Mirault et al [14]. Furthermore these observations provide a reasonable basis for interpretation of the heat-shock protection phenomenon described by Milkman several years ago [3, 4]. Milkman and collaborators demonstrated that when Drosophila pupa (24 hours) were subjected to a mild heat shock prior to one that would be lethal alone, some animals would survive. They also reported that a pretreatment of this kind would prevent the induction of a phenocopy of the mutant crossveinless.

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The work described in this paper was carried out to evaluate the molecular basis for the phenomenon of protection by pretreatment by a mild heat shock, and to gain additional information on possible functions of the heat-shock proteins. We feel that the present evidence supports the hypothesis that the heat-shock proteins are involved directly in storage of mRNAs which in turn account for the protection effects.

MATERIALS AND METHODS

Fly Stocks

A single Oregon R wild stock was used for all the experiments described in this paper. This stock is a little more heat resistant than the Ore-R, Urbana S, and Canton-S cultures used previously. In this case 41.3°C for 40 minutes is about equivalent in survival to the 40.2°C for 40 minutes applied in earlier eperiments [1].

Details of culture and selection of animals in developmental synchrony were described earlier [1, 5].

Heat Treatments

All heat shocks were given by immersion of samples contained in vials in a water beth regulated to ± 0.1°C. Regulation to at least this precision is essential at the higher temperatures. Vials contained moist paper to maintain a high humidity.

Labeling

Labeling of salivary glands was carried out at 25°C in MOPS buffer at pH 7.0 [6] using using three pairs each in 4 μl of ^{35}S-methionine (Sp activity 600 Ci/mM) or ^{3}H-uridine (Sp activity 50 Ci/mM), each containing 10 μCi.

Gel System

The SDS acrylamide gel used for these experiments was a 10–20% exponential gradient gel prepared as described by Van Blerkom and Manes [7] and modified by Moller [8].

Scanning Electron Microscope

Samples for observing bristle morphology were prepared as described by Mitchell and Lipps [1], and those for observing chromosome autoradiographs were made as described earlier [1, 9].

RESULTS

Protection for Survival

As shown in Figure 1, third instar larva are all killed by a heat shock of 40.5°C for a period of 20 minutes. However, if the animals are pretreated in the temperature range of 31–37°C and then subjected to the lethal temperature, as many as 50% of the animals survive and develop normally. Some protection is evidenced at temperatures as low as 28°C and as high as 39°C.

The specific conditions for lethality and protection shown for third instar larva (Fig. 1) do not apply to animals at all ages and stages of development, but the survival-protection effect does obtain at all stages tested and to cell cultures. Some specific examples are shown in Table 1. These data show the generality of the pretreatment effect

down in the cell level and also the relatively greater resistance of pupa (pharate adults) to heat shock. The latter was defined more extensively in connection with earlier work on phenocopies [10].

The data of Figure 1 and Table 1 suggest that mild heat pretreatments cause the accumulation of substances which serve to rescue flies and cells from death due to a sudden high temperature heat shock. It also appears, as shown in Figure 1 and later in Figure 4, that a maximum protective effect is achieved within the first 30 minutes of the pretreatment regardless of the specific temperatures and tissues involved.

Prevention of the Hook Phenocopy

In an earlier paper [1] we described a series of stage-specific phenocopies produced by heat shock of pupa in the 30–50 hour range. Here we have made use of this series to answer the question of whether pretreatments are effective in preventing a specific defect. As shown in Figure 2, pretreatment does indeed prevent the production of the specific phenocopy of the mutant hook, since the normal scultellar bristles shown in part A were present on all of the animals treated for 40 minutes at 35°C followed by 30 minutes at 41.3°C, while the hook types shown in part B appeared in all of the flies from the single treatment at 41.3°C for 30 minutes. This effect is as decisive and dramatic as that shown by the effect of pretreatment on survival (Fig. 1), and it could be due to the same general phenomenon. However, a high degree of specificity is shown in this case as demonstrated by the data summarized in Figure 3, parts A and B. In part A it is shown that a pretreatment about 4 hours before the critical period for production of the hook phenocopy [1] did not affect the subsequent induction of this particular phenocopy. Actually, the critical period came a little sooner than in the control, as might be expected from the elevation of the temperature during the pretreatment. The second series, beginning with a pretreatment around 32 hours (1–2 hour before the critical period), resulted in some reduction of the frequency of hook phenocopies. In contrast the pretreatments given at 34 hours and at 35.5 hours (both within the hook sensitive period), gave essentially total protection when the high-temperature shock was given immediately after the pretreatment. It is important to note that in both of these latter cases a significant number of phenocopies did appear when the second shock was delayed for an hour

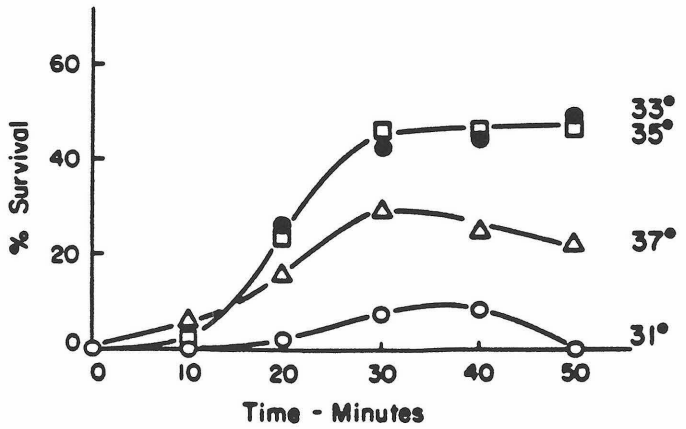


Fig. 1. Survival of larvae following heat shocks. Mid-third instar larva were pretreated for the periods of time at the temperatures shown on the graph and all animals were given a heat shock of 40.5°C for 30 minutes immediately after the pretreatment. Animals were maintained at 25°C before and after the heat treatments. Survival is given as adult flies.

TABLE 1. The Effects of Some Pretreatment Conditions on Animals at Different Stages of Development and on Cell Cultures

Material	Pretreatment	Shock	Survival %[b]
Larva 5 day	25°C	40.5°C 30 min	0
	35°C 50 min	40.5°C 30 min	37
Prepupa 2 hr	25°C	40.5°C 20 min	0
	34°C 60 min	40.5°C 20 min	38
Pupa 36 hr	25°C	41.3°C 30 min	22
	34°C 60 min	41.3°C 30 min	73
Adults 2 day	25°C	40.5°C 25 min	16
	35°C 60 min + 25°C 60 min	40.5°C 25 min	89
Adults 7 day	25°C	40.5°C 25 min	0
	35°C 60 min + 25°C 60 min	40.5°C 25 min	43
Cell culture[a]	25°C	40.5°C	0
	35°C 60 min	40.5°C	75

[a]The cell culture line was derived from the Ore-R stock as described by Petersen et al [27]. Data are given as percent of normal growth rate.
[b]Survival to adult flies.

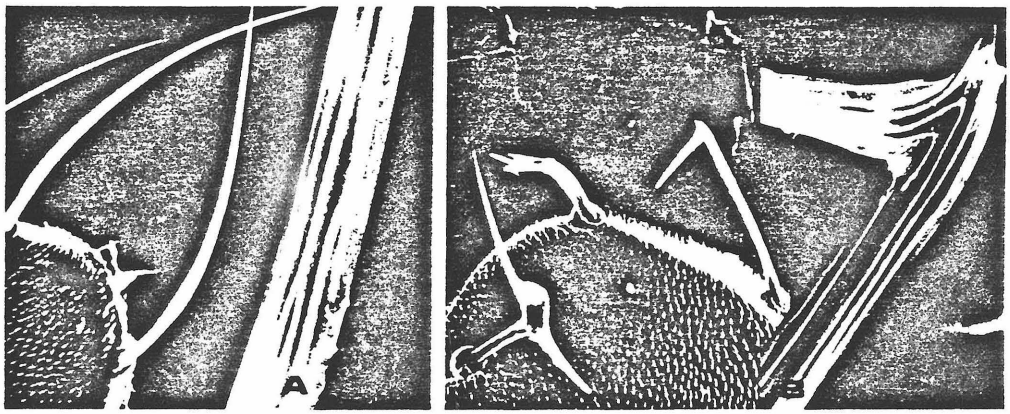


Fig. 2. Prevention of phenocopy production by a mild heat shock. Pupa synchronized in developmental time to 36 ± 1 hour after puparium formation were: A. Treated at 35°C for 40 minutes and then at 41.3°C for 30 minutes. B. Treated at 41.3°C for 30 minutes. All animals were kept at 25°C before and after the heat treatments. The photographs (scanning electron microscope) show the scutellum and bristles from an adult fly and a higher magnification of one bristle portion in each case. All adult flies from the pretreated (A) group (17) had normal bristles and all (21) adult flies from the (B) group had abnormal scutellar bristles at least as extreme as those shown.

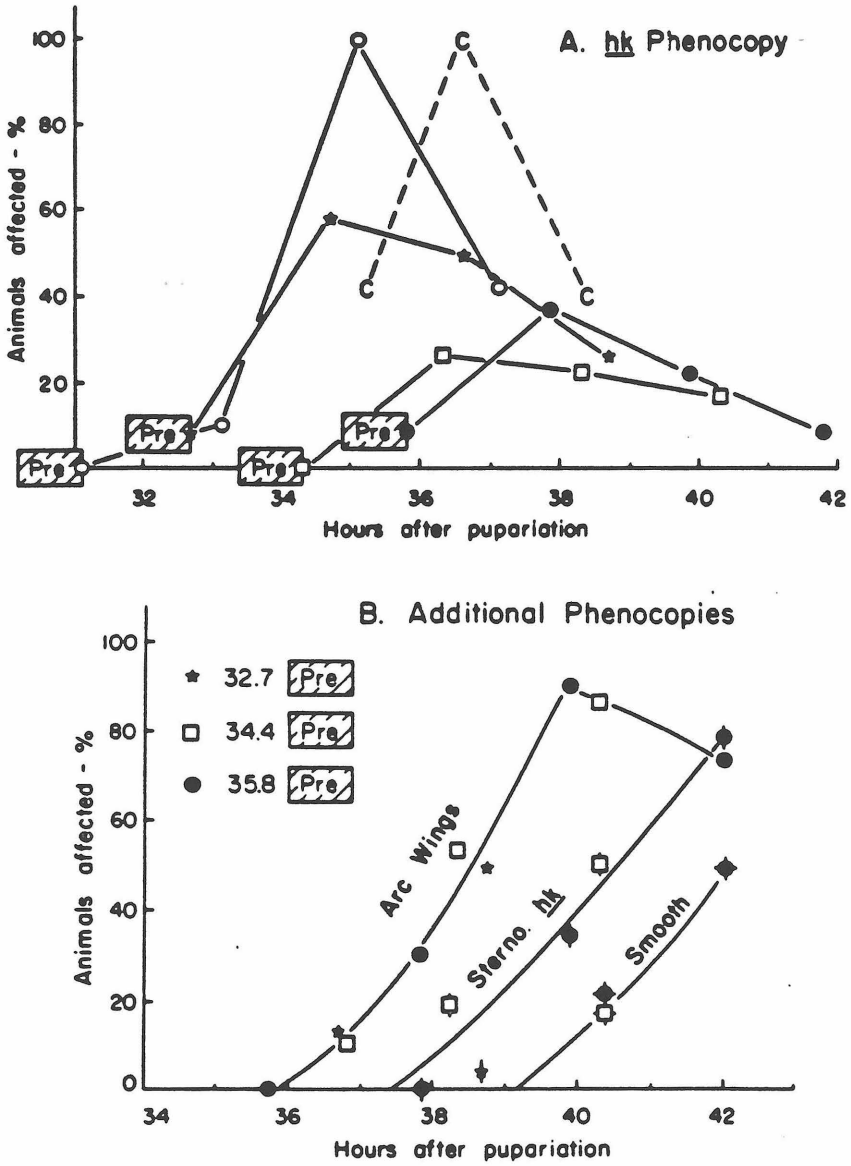


Fig. 3. Specific effects of pretreatment on phenocopy production. A. The hook phenocopy: The shaded blocks labeled "pre" indicate the developmental time at which a pretreatment of 34°C for 40 minutes was given to a synchronous group of 160 animals. In each case 40 pupa were heat shocked immediately after the pretreatment. The remainder were kept at 25°C and 40 pupa were heat shocked subsequently at about 2, 4, and 8 hours after the pretreatment. The pupa were then kept at 25°C until eclosion and the adult flies were scored for the hook phenotype. The dashed line labeled C shows three points for a control set which was not pretreated at 34°C. The entire experiment was done with the same synchronous collection of pupa (760 animals). B. The same animals scored for hook frequency in part A were scored for the phenocopies arc wing, sternopleural hook, and smooth bristles. The symbols used refer to the time of pretreatment. The points for sternopleural hook have a vertical line through the symbols and the points for smooth bristles have both a vertical and a horizontal line.

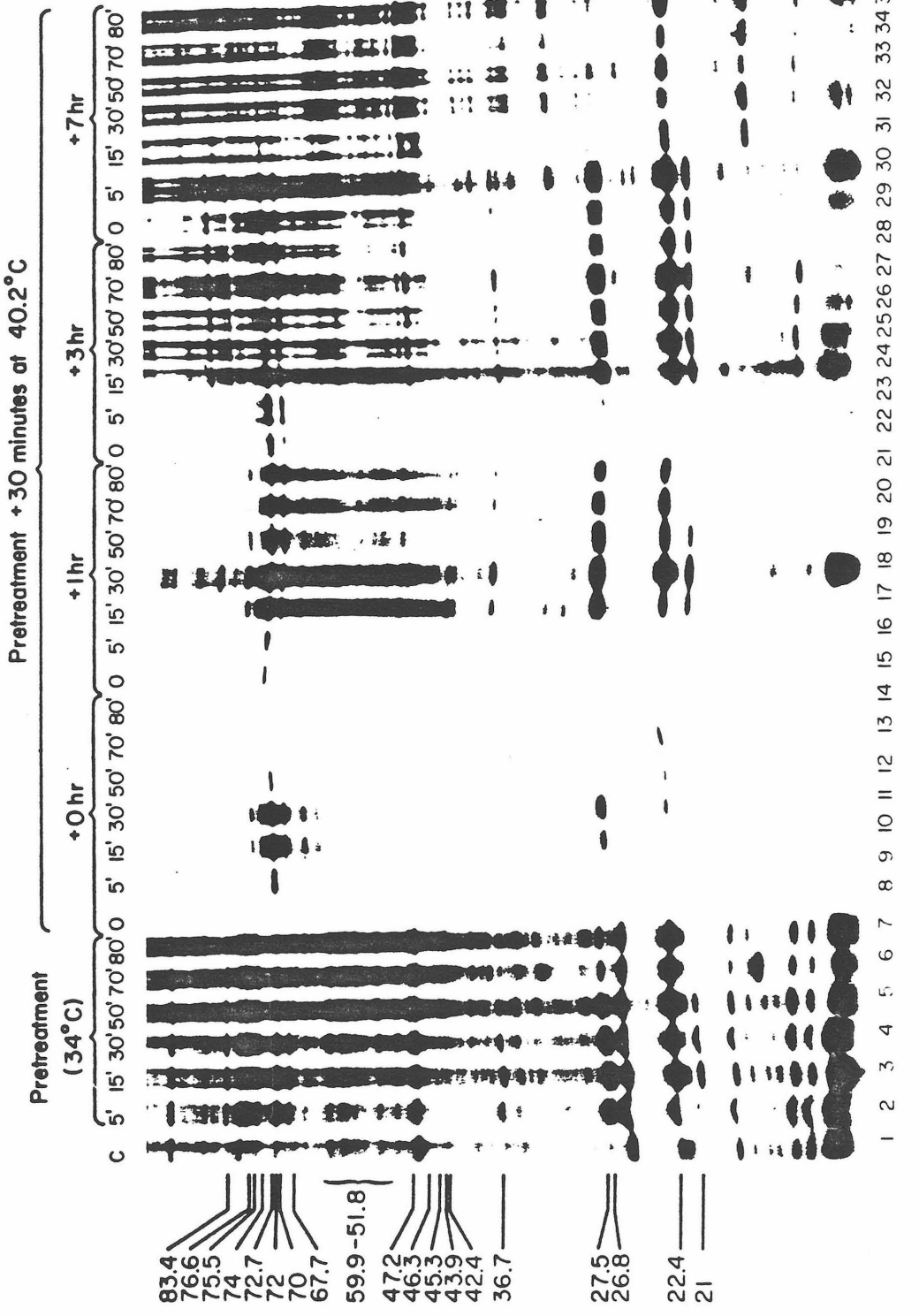
after the pretreatment. This shows that the protective agent specific for hook is labile. The data shown in Figure 3B provide additional and essential support to the foregoing conclusions. These results are from the same animals scored for the hook phenocopy shown in part A. They demonstrate that the phenocopies sternopleural hook, arc wings, and smooth bristles appeared on schedule regardless of when the pretreatment was given. This supports the contention of independent events giving rise to independent phenocopies as stated earlier [1].

Protection and Protein Synthesis

As shown in detail in Figure 4, the protection phenomenon is as clearly illustrated in terms of protein synthesis patterns as it is by morphological criteria. The data presented are from an experiment in which animals were treated in various temperature sequences and then their salivary glands were dissected and pulse-labeled with ^{35}S-methionine. In the first set of samples (at the left in Figure 4, tracks 2–7) larva were heated at 34°C only, followed by 20 minutes labeling at 25°C. It is clear that the most prominent heat-shock proteins (labeled at the left as 83.4, 72, 67.7, 36.7, 27.5, 26.8, 22.4 and 21) appear promptly, even with the shortest treatment at 34°C, and they reach a maximum by 30 minutes. However, most of these do not appear as prominent components after 50 minutes at 34°C. It is of special interest to note that most of the normal components (track 1) are synthesized throughout the 80 minutes at 34°C. It appears that this concomitant synthesis capacity for normal and heat-shock proteins may be essential to the protection phenomenon.

The remaining four sets of samples (tracks 8–35) in Figure 4 show the effects of a second heat shock (40.2°C for 30 minutes) on a series of samples pretreated as in the series illustrated in tracks 1–7. All samples were given the higher temperature shock immediately after the pretreatment but then they were kept at 25°C for: 0 hour (tracks 8–14); 1 hour (tracks 15–21); 3 hour (tracks 22–28); and 7 hour (tracks 29–35), before dissection and labeling. Thus these sets of samples show the immediate effects of the high-temperature shock on translational capacities and the effects of pretreatments on the recovery of translational capacities. First, in the 0 hour series there is a dramatic reduction in all protein synthesis but protection is evident in the 5–50 minute range, with both normal and heat-shock proteins showing strongly at 15–30 minutes (tracks 10 and 11). However, it is just as remarkable that relatively little translation is evident in the 50, 70, and 80 minute samples. If, for example, one compares the protein synthesis exhibited in tracks 4 and 5 with the corresponding samples in tracks 11 and 12, it would appear that the second and higher temperature shock caused either much more degradation of messenger RNA in the 50-minute sample than in the 30-minute sample, or much

Fig. 4. Effects of pre-heat shocks on protein synthesis in salivary glands. Each sample of the 35 shown (numbers at the bottom) was prepared for electrophoresis on a gradient SDS gel from three pairs of salivary glands dissected from late third instar larva. The glands were labeled for 20 minutes at 25°C immediately after the larva had been subjected to the various heat treatments. Samples 1–7 were heated for 0.5, 15, 30, 50, 70, and 80 minutes at 34°C only. Samples 8–18 were heated for the times indicated at 34°C and then shifted to 40.2°C for 30 minutes before labeling. Samples 15–21 were treated as lanes 8–14 except that they were allowed to recover 1 hour after the second heat shock before labeling. Samples 22–28 and 29–35 were allowed to recover from the second heat shock 3 hours and 7 hours respectively before labeling. The numbers at the left refer to the molecular weights ($\times 10^3$) of the more than 20 heat-shock proteins observed on this and additional gels (Moller, unpublished). The picture presented here is from a single gel which originally contained also samples from 40 and 60 minute treatments in each set. These were removed to make the final autoradiograph less cumbersome. In all cases these patterns were very similar to the 50 minute samples in each set.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Pretreatment (34°C)
+0hr
+1hr
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83.4
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72
70
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59.9-51.8
47.2
46.3
45.3
43.9
42.4
36.7
27.5
26.8
22.4
21

4

more of the 50-minute messenger was rendered inaccessible to translation. The same is true for the 70- and 80-minute samples. Degradation of the messenger seems unlikely from a consideration of the results shown in the 1-hour and 3-hour series. Here, the 50-80-minute samples (tracks 19–21 and 26–28) show a reappearance of strong synthesis of the major heat-shock proteins. These results require either extensive new transcription in the 1- and 3-hour series, which is unlikely as shown earlier [1, 2], or reactivation of the message stored in a masked form during the pretreatment phase at 34°C.

Protection and RNA Synthesis

We presented earlier evidence [1] that a high temperature (41.3°C for pupa) shuts down both translational and transcriptional activities. It was further shown that in this situation protein synthesis resumes before general RNA synthesis. These experiments were done on thoractic hypoderm tissue from 34-hour pupa and the results anticipated the suggestions made here regarding the protein synthesis patterns in Figure 4. That is, the initial resumption of protein synthesis after it has been turned off by a heat shock can be accounted for if the conditions favor storage of mRNA in a form that can be reactivated. Additional evidence that supports this conclusion is shown in Figure 5. Parts B, C, and D of Figure 5 are from autoradiographs (scanning electron microscope) of the tip of the left arm of chromosome 3 (as a representative region) as shown in part A. Each chromosome portion was identified before the autoradiography by a photograph as shown in part A. Parts B, C, and D are displayed as viewed by the scanning electron microscope, since silver grains are not obscured by the banding-pattern background when this method is used. The preparations were made from a 10-minute pulse-labeling of late third instar salivary glands of Drosophila melanogaster and the conditions used were like those described for the protein synthesis results shown in Figure 4. Specifically, picture D at the right corresponds to the 25°C control in track 1 (Fig. 4). This general distribution of silver grains is typical for a chromosome region where there are no prominent puffs [11]. Picture C is from pulse-labeled salivary glands from larva given a heat shock of 40.2°C for 30 minutes (Fig. 4, track 15). Picture B is from glands from animals given a 50-minute treatment at 34°C, followed by a 30-minute shock at 40.2°C, and then a 60-minute incubation at 25°C prior to the pulse label (Fig. 4, conditions for track 19).

The crucial point to note in Figure 5 is that the labeling patterns which visualize transciption directly are virtually identical in pictures B and C. In sharp contrast, the corresponding translational activities (Fig. 4, tracks 19 and 15) are very different with extensive translation in the pretreated sample and very little in the other. Nearly all of the transcriptional activity that is observed in B and C (Fig. 5) is in the heat-shock puff region 63 BC as marked, but the total label in each is still less than 5% of that in the control (D) which does not include a heat-shock puff. Thus the protein synthesis observed in the pretreated samples must be derived from the stored message.

DISCUSSION

We previously demonstrated [1] that both RNA and protein synthesis are turned off by a drastic heat shock to Drosophila tissue. Furthermore, it was clearly shown that in the recovery process protein synthesis resumes to the normal level long before transcription is reactivated. These conclusions are confirmed for milder conditions by results described in this paper where RNA synthesis was observed directly from autoradiographs of salivary-gland chromosomes (Fig. 5), and protein synthesis was observed by autoradiography of pulse-labeled proteins on acrylamide gels (Fig. 4). In view of these facts it

is possible to evaluate mRNA pool compositions by pulse-labeling of proteins after a heat shock. Thus, the data such as those presented in Figure 4 demonstrate heat-shock effects on protein synthesis but they also reflect the nature of the mRNA pool and they may show selective translational activities.

Protein Synthesis and Protection for Survival

Experiments described in this paper and earlier [1, 2, 9, 13] suggest that the heat-shock proteins are themselves involved in protection for survival by pretreatment. This possibility is supported by the following facts:

1) In all conditions of recovery after heat shock, synthesis of the induced heat-shock proteins occurs first even though messages for non-heat-shock proteins are present (Fig. 4) [1, 2].

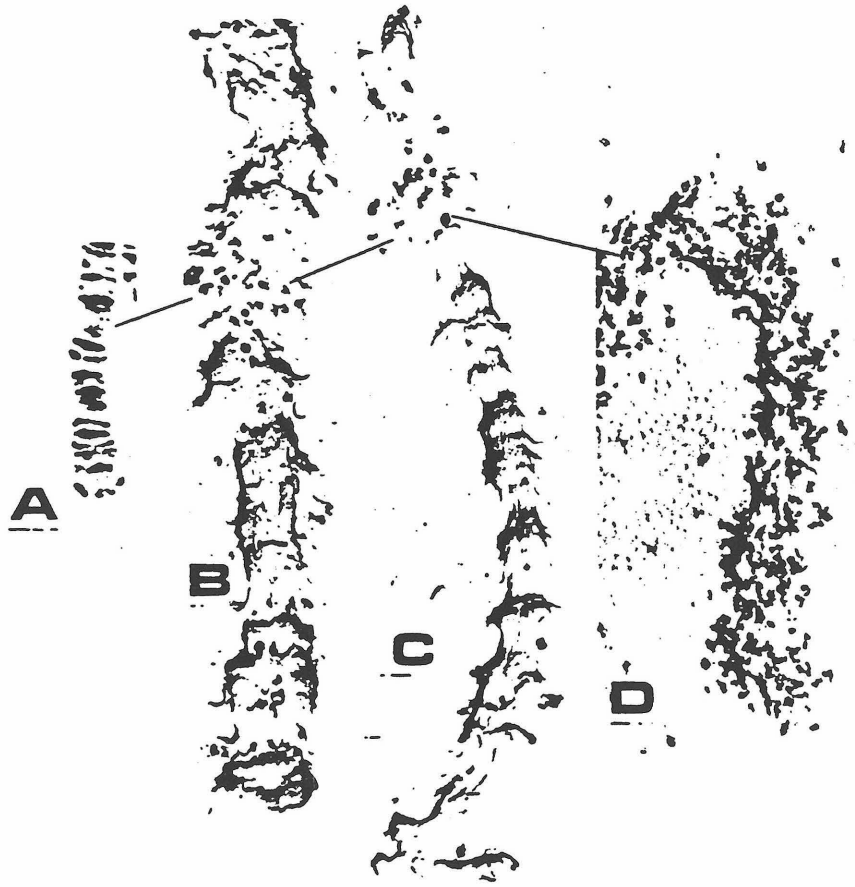

Fig. 5. Transcriptional activity on salivary gland chromosomes. A. Shows a light microscope picture of the tip of a normal 3 L salivary gland chromosome while B, C, and D show autoradiographs of corresponding chromosome sections as revealed in the scanning electron microscope photographs. The connecting line shows the position of the heat-shock puff designated 63BC. B. An autoradiograph from a gland from a larva which received a 50 minute pretreatment prior to a 30 minute shock at 40.2°C. C. An autoradiogram from a gland from an animal which received a 30 minute heat shock at 40.2°C. D. An autoradiogram from a gland from an animal which was kept at 25°C. All labeling was done for 10 minutes at 25°C using ^{3}H-uridine and isolated salivary glands.

2) The minimum time required and the optimum temperature needed to obtain the best protection for survival (of larva for example, in Fig. 1) corresponds closely to the condition of pretreatment which simultaneoulsy gives the greatest production of both heat-shock and non-heat-shock proteins. This is the condition shown in Figure 4 where the effects of time of pretreatment on protein synthesis are shown. With respect to temperature, protection is less at both lower and higher temperatures (Fig. 1) than in the 34–35°C range and the protection and protein-synthesis patterns correlate extremely well [13 and unpublished data]. That is, both protection and production of heat-shock proteins diminish to a nonsignificant level below 30°C and both protection and non-heat-shock protein production diminish to a very low level about 39°C.

3) Both induction of heat-shock proteins and protection are induced by the same treatment in cell lines and in animals at all stages of development. These various facts show different kinds of correlations between the presence of heat-shock proteins and protection phenomena. They suggest but do not prove that the heat-shock protein(s) is directly involved physically with the storage of mRNA and/or the regulation of protein synthesis just as they have been shown to be involved directly with salivary gland chromosomes [9]. There now seems little doubt that heat shock does affect storage of the message and preferential translation of the message after heat shock is well established [12, 13, 14]. The preferential translation also shows clearly in Figure 4 after the double heat shock (see the 30-minute samples at 0 and 1 hour).

Phenocopy Prevention

Evidence has been presented [1] which suggests that a particular phenocopy results from the absence of a particular translation product which should be produced at the right time in a developmental sequence. Since the conditions required for the hook-phenocopy induction prevent transcription but not translation for the complete sensitive period [1], it is reasonable to think that the pretreatments which protect against the phenocopy (Figs. 2 and 3) simply promote storage of a particular mRNA. Thus, following the heat shock during the sensitive period, such accumulated message can be used to produce a particular protein when it is useful in the construction of a normal bristle. By this interpretation, pretreatment for survival protection and for phenocopy prevention are simply different manifestations of the same phenomenon; that is, the storage of mRNAs, somehow, under the influence of heat-shock protein.

A Speculative Model

It now appears that the molecular basis of phenocopy formation and protection, as a result of heat shock, may be understood most clearly as part of a general pattern of dynamic interactions which involve simultaneously transcription, translation, and translation products. A speculative picture which fits the present information remarkably well is shown in Figure 6. In this pattern, beginning at the chromosome level, we envisage that the preheat treatment induces production of heat-shock messages in addition to the normal complement that is already in production. Then, all of these messages are exported as ribonucleoproteins (RNPs) to the cytoplasm where they can go to polysomes for translation or enter into an inactive pool. Finally, as increased amounts of heat-shock protein(s) is synthesized, transcription is more and more repressed and more and more messages are sequestered in the RNP pool. It is this gradual process which is essential in the pretreatment, since a sudden shock at a high temperature represses all transcription quickly without allowing message accumulation. The accumulated and inactive message would

provide the basis for survival after a drastic shock and it is reasonable to think that the message pool provides the memory for resumption of development in the pattern that existed when the heat shock was applied.

Although the overall pattern in Figure 6 is speculative, various presumptions have a sound basis in the facts presented earlier [1, 9] and here in Figures 3–5. In addition, the picture is not greatly different from patterns implied from the work of a number of investigations [16–19, 24]; and the reviews by Perry et al [20], Lewin [21], and Spirin [22]. These are concerned with the composition and potential functions of ribonucleoproteins, and the most pertinent in relation to our results is the earlier report of Schochetman and Perry [25] in which an apparent direct relation was shown between heat treatments and RNP accumulation in L cells. The evidence presented here supports both the existence of a cytoplasmic pool of RNPs and the postulate that such RNPs are fundamental importance in the regulation of development. We have added the suggestion of a direct involvement of heat-shock protein(s) in the system both in the nucleus [9] and in the cytoplasm, but this may be only part of the normal picture. It is of interest also to note that the heat-shock phenomenon (the induction of synthesis of specific proteins) has now been observed in a variety of cell-types other than Drosophila [23, 26]. These include HeLa cells, chicken embryo fibroblasts, BHK cells, and mouse L cells. No information is yet available on the question of whether the protection by pretreatment obtains in these cases.

ACKNOWLEDGMENTS

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We are indebted to Dr. Edward Berger of Dartmouth College for a reminder of the early work of the Milkman group. We are indebted to Carol Shotwell for technical assistance during some of this work.

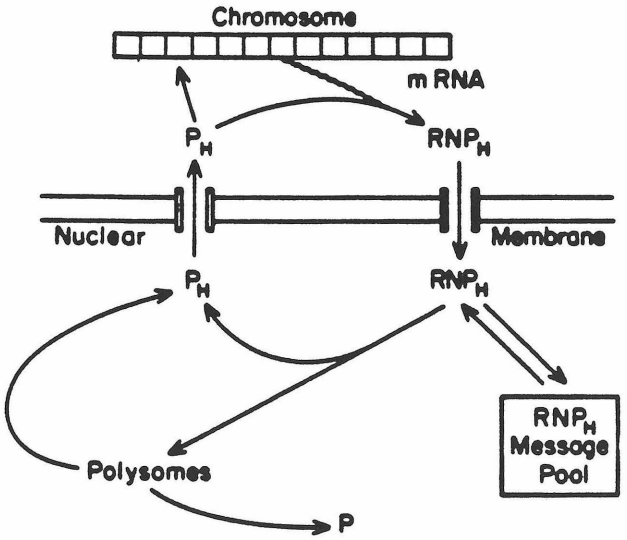

Fig. 6. A diagram to illustrate a dynamic mechanism that may explain the pretreatment-protection phenomen. P_H is used to indicate one or more heat-shock proteins and RNP_H ribonucleoproteins in which heat-shock proteins are involved. It is not intended that the heat-shock proteins exclusively are involved in the transport cycle or in maintaining a message pool.

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Appendix

Preliminary Studies on Identification of Genes Coding for Developmentally Stage-Specific Proteins

INTRODUCTION

Puffs in the salivary gland polytene chromosomes were classed as either developmentally specific or developmentally unspecific (1). The latter class was not necessarily active continuously but they did not change their activity in any regular way as development proceeded.

Certain morphological events are correlated with particular puff stages during normal development, i.e., secretion of the salivary gland glue into the lumen occurs after puffing stage 4 and puparium formation occurs at puffing stage 10/11 (2). Ashburner (3) noted that peak periods of puffing activity occurs at the time of puparium formation and in 8-hour prepupae. The "prepupal molt" occurs 4 hours after puparium formation and the true pupal molt occurs 12 hours after puparium formation. In contrast to the other puffs being active at only one time, many puffs are common to both of these periods yet inactive during the intermolt. The induction and regression of puffs common to both periods may differ according to the developmental stage analyzed and implies proposals for a cascade type of control mechanism operating on puffs (4).

Newly activated puffs appear to contain especially large quantities of "puff proteins". During puff induction, the accumulation of puff proteins precedes any visible change in chromosome structure and detectable RNA synthesis (5,6,7). Mitchell and Lipps (8) had demonstrated that protein accumulation occurs at a specific puff site just an hour before it is due to puff. Experiments conducted by Tissières et al. (9) showed that the protein patterns in the salivary glands are modified when the glands are subjected to a heat treatment at 37°C for 20 minutes. This change in pattern is accompanied by changes in the puffing pattern. Further investigations on the role of protein synthesis in the induction of puffs were done by Mitchell and Lipps (8) and demonstrated identical patterns for nuclei and whole cells of heat-shocked animals but different patterns in non-heat shocked animals. These differences

could be due to induction or regression of new puffs or could be due to a rapid exchange of proteins between the nucleus and the cytoplasm.

The synthesis of salivary glue glycoprotein has been correlated with the presence of intermolt puffs in the salivary gland chromosome. The structural genes for four of the polypeptides were located in the intermolt puff 68C and 3C (10).

The process of pupation is another morphological event which could be attributed to specific puff stages. The experiments in Chapter 1 clearly demonstrate active rates of synthesis for a set of low molecular weight proteins during the prepupal stage and a dramatic reduction in synthesis at the time of pupation. The transfer of these small polypeptides to the pupation fluid was also demonstrated in these studies. Although the possible role in pupation of these small polypeptides remains speculative, it would be of interest to identify the structural genes for these proteins and to be able to correlate them to specific puff sites.

MATERIALS AND METHODS

Fly Culture

Oregon-R wild stock of Drosophila melanogaster grown in mass culture (11) was used for the experiments. Five-hour pupae were selected in developmental synchrony to ± 1 hour by the flotation method (11) and kept in a petri dish at 25°C for a period of one to three hours. White prepupae were selected when puparium formation started to within 0.5 hour.

RNA Preparation and Translation

452 pairs of salivary glands from 6-9 hour prepupae and 82 pairs of salivary glands from white prepupae were dissected and ground with buffer and phenol (25 µl each) as described by Petersen and Mitchell (12) with modifications (S. Falkenthal, personal communication). Samples were extracted with phenol and chloroform (1:1) 4-6 times and re-extracted with chloroform three times. Precipitation and reprecipitation were done by adding 3 volumes of ethanol containing 1 M NaAc. The

RNA precipitate was washed with 70% ethanol and redissolved in 20 µl of water. The RNA content of the supernatant was determined spectrophotometrically.

Translations were done using the New England Nuclear reticulocyte kit system as described by Mitchell and Petersen (13).

Gel Electrophoresis and Autoradiography

Translated samples were applied on SDS-polyacrylamide slab gels containing a linear gradient of polyacrylamide and bisacrylamide as described previously (14). The gels were then dried and exposed for autoradiography on Kodak X-AR 5 X-ray film and exposed at room temperature for 4-6 days.

Preparation of Hybridization Probes

3' specific complementary DNA (cDNA) were synthesized in 30 µl reactions containing 10 µg of RNA according to the procedure described by Mullins et al. (17). Two different cDNA probes were prepared by using RNA from 6-9 hour prepupae and RNA from late larvae.

Differential Plaque Filter Hybridization

A Canton-S Drosophila recombinant DNA library in Charon 4 was used for the differential plaque hybridization. The library, prepared by J. Lauer, was constructed by inserting randomly sheared Drosophila embryonic DNA (12-20 kb) terminated by synthetic Eco R1 linkers into bacteriophage Charon 4. Recombinant phage were plated and multiple filter replicas were prepared sequentially from each plate as described by Benton and Davis (15) and St. John and Davis (16). The replica filters were separately hybridized to the stage-specific hybridization probes. Hybridization, filter washing and autoradiography was as described by Mullins et al. (17).

DNA Extractions ("mini-prep")

Attempts to extract DNA from the stage-specific clones were done using two different procedures.

One procedure was adapted from the "mini-prep" procedure of Ish-Horowicz

and Burke (19) with modifications by S. Falkenthal (unpublished). Each of the 15 clones was plated on 1.5% agarose plates and incubated at 37°C until confluent lysis occurred (9-10 hours). The plates were then placed at 4°C for 1 hour before the addition of 5 ml of 10 mM Tris, pH 7.4, 2 mM $MgSO_4$ to each plate. The plates were then incubated at 4°C for approximately 12 hours and the solution was decanted from each plate. This was followed by the addition of RNase and DNase to a final concentration of 1 µg/ml of each. The samples were allowed to incubate for 2 hours at 4°C and then incubated at 68°C for 15 minutes after the addition of 0.5 ml of 1.6% SDS, 160 mM EDTA, 800 mM NaCl. Proteinase K was added to a final concentration of 50 µg/ml and the samples were incubated for 2 hours at 37°C. The DNA was extracted with phenol:chloroform:isoamyl alcohol (50:48:2) until there was no material at the interface. The solution was re-extracted with chloroform: isoamyl alcohol (24:1) twice and the DNA was precipitated with an equal volume of isopropanol. The DNA was reprecipitated and washed with 70% ethanol and resuspended in 100 µl of 10 mM Tris, pH 7.4, 1 mM EDTA. For the enzyme digestions, 20 µl of DNA samples were used.

Another method of DNA extraction that was attempted was the procedure described by Thomas and Davis (20) wherein the DNA is isolated as a band in a cesium chloride gradient.

Restriction Endonuclease Digestion and Gel Electrophoresis

Restraiction endonucleases were obtained from New England Biolabs and the digestions were carried out as described elsewhere (17). The digested samples were applied on 1% agarose gels set up in 18mm x 14mm x 2.5mm slabs.

Hybrid Selection

RNA was selected by a procedure similar to Ricciardi et al. (21). Bacteriophage DNA was denatured and immobilized onto nitrocellulose filters. 100 µg of total RNA from 6-9 hour prepupae was hybridized to all of the filters simultaneoulsy.

Following the hybridization, the RNA was eluted, translated and the translation products were electrophoresed on SDS-polyacrylamide gels.

RESULTS

Translation of total RNA from different developmental stages showed the presence of a low molecular weight component in 6-9 hour prepupae which is not present in the late larvae. However, it is difficult to ascertain whether this component corresponds to any one of the four specific polypeptides mentioned in Chapter 1. Based on these results, it was assumed that hybridization probes synthesized from the stage-specific RNA could be used to select clones containing developmentally regulated sequences. Initial screening of the Drosophila genomic DNA library yielded 36 clones complementary to the cDNA from 6-9 hour prepupae which did not hybridize to the late larval cDNA. Re-screening of these 36 clones with the two different cDNAs revealed 15 clones which only hybridized to the cDNA from 6-9 hour prepupae. A representative example of this is shown in Figure 1.

DNA was extracted from each of these 15 clones and digested with Eco R1. Gel electrophoretic patterns of the digested samples are shown in Figure 2. The result indicates that the clones are different, however, this does not mean that they represent 15 distinct DNA regions because the Drosophila genomic library used a random shear library. A more definitive comparison can be obtained by digesting with a number of restriction enzymes simultaneously.

The cloned DNAs were subsequently immobilized on nitrocellulose filters and hybridized to total RNA from 6-9 hour prepupae. Translation of the hybrid-selected RNAs which were eluted from the filters did not show any conclusive results. There was no detectable component in the molecular weight region of the small polypeptides present in the salivary glands. It was not possible to detect the presence of a precursor because no antibody to these components was available. It should be noted that there were very discrete differences among the translated products in the higher

molecular weight region (Figure 3). A possible explanation for the negative result is that the amount of RNA which was eluted is below the level required for translation. The amount of RNA in each sample was too low to be measured spectrophotometrically.

The different procedures for extracting DNA did not yield sufficient DNA to enable the preparation of a probe for subsequent use in in situ experiments which were planned in order to correlate a specific puff site with any one of these low molecular weight proteins found in the salivary gland.

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Figure 1. Selection of clones containing developmentally-regulated sequences. Complementary DNAs were synthesized to RNA from 6-9 hour prepupae and to RNA from late larvae. There were 36 clones obtained which hybridized to cDNA from 6-9 hour prepupae which did not hybridize to cDNA from late larvae. Re-screening of the 36 clones with the cDNA from the two developmental stages yielded 15 clones which hybridized to the cDNA from 6-9 hour prepupae alone.

Prepupae (6-9hr) Late Larvae

Figure 2. Eco R1 digestion of DNA from the 15 stage-specific clones. DNA was extracted from the stage-specific clones by the "mini-prep" procedure and subsequently digested with Eco R1. The digested samples were then applied on 1% agarose gels. The numbers above each lane indicate the designated number of each stage-specific clone. The left lane is a restriction digest of lambda which was used as a size marker.

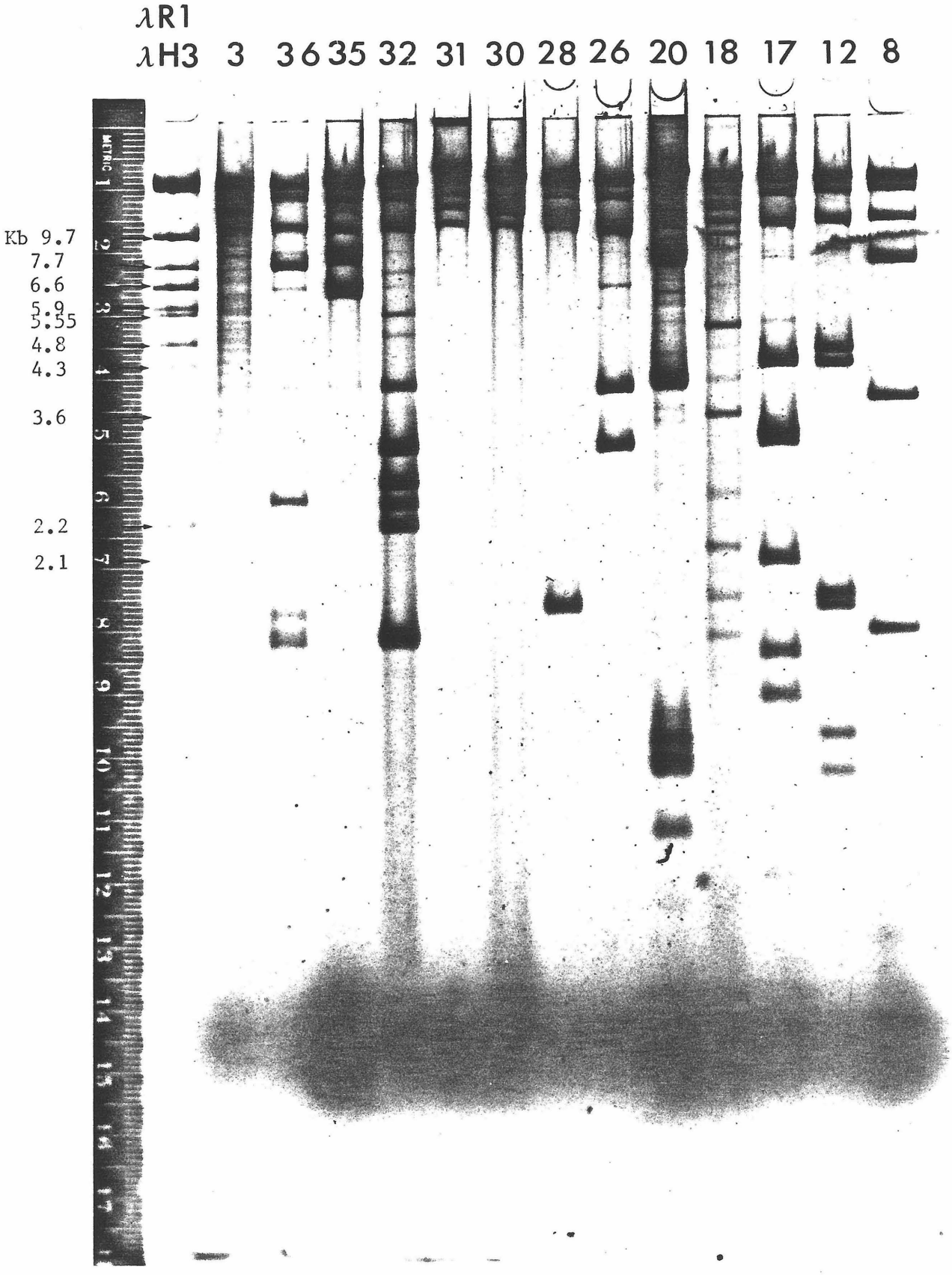
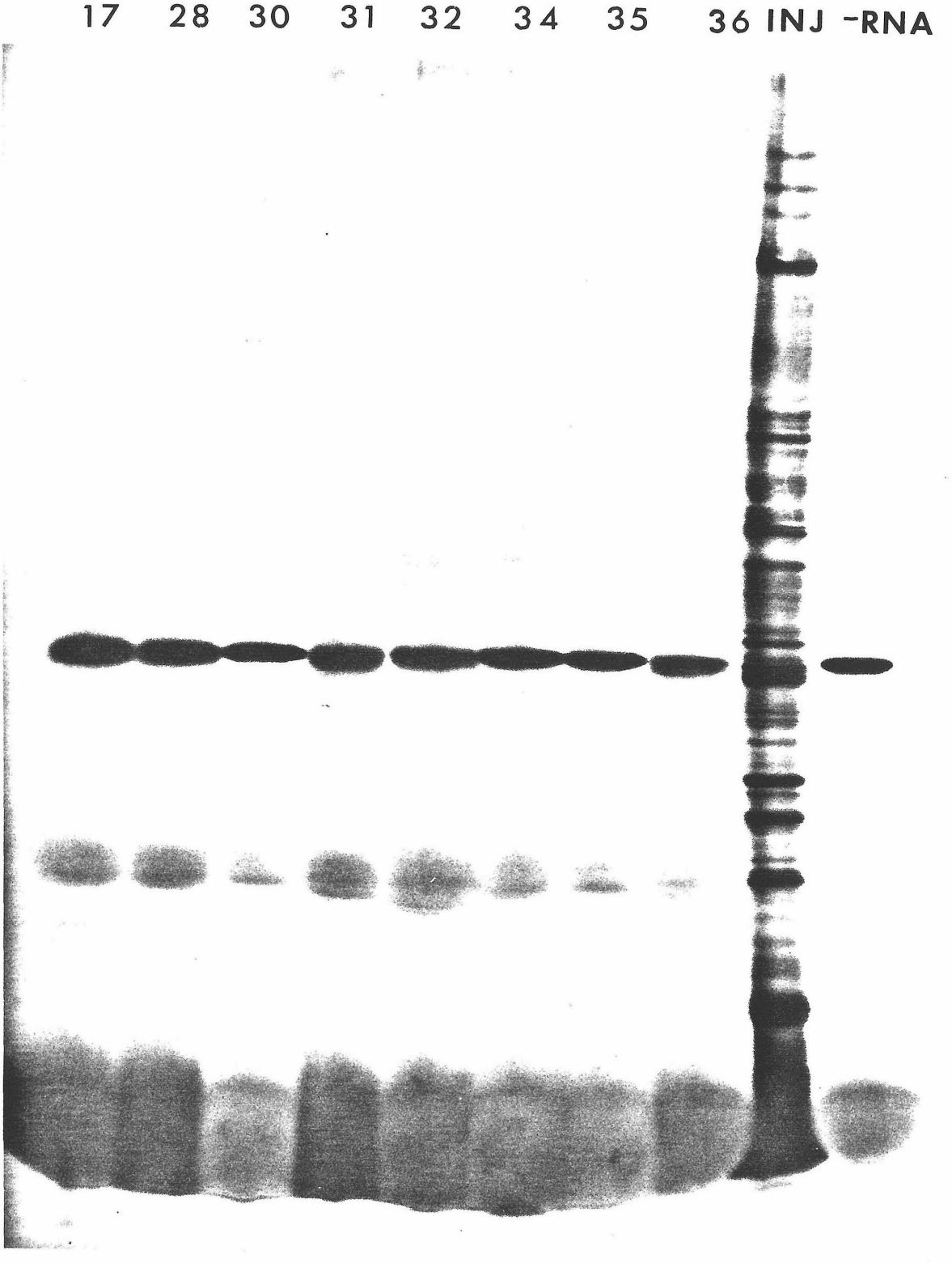

λR1
λH3 3 36 35 32 31 30 28 26 20 18 17 12 8
Kb 9.7
7.7
6.6
5.9
5.55
4.8
4.3
3.6
2.2
2.1

Figure 3. Translation products of the hybrid-selected RNAs. Bacteriophage DNA was denatured and immobilized onto nitrocellulose filters and RNA from 6-9 hour prepupae was hybridized simulataneously. The hybrid-selected RNAs were translated and applied on an SDS-polyacrylamide gradient gel.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