STUDIES ON PROTEIN SYNTHESIS AFTER HEAT SHOCK IN *DROSOPHILA MELANOGASTER*

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To my father, Michael

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Abstract

The synthesis of the heat shock proteins on cytoplasmic rather than mitochondrial ribosomes has been shown. The time course of the synthesis of heat shock proteins in prepupal and pupal stages of Drosophila melanogaster has been analyzed. Prepupae were heat shocked at 37.5°C for 20 minutes and pupae, either at 37.5°C for 20 minutes or at 40.2°C for 40 minutes. In prepupae, all of the heat shock proteins are synthesized immediately after the shock and continue to be synthesized for two to three hours. By three hours after the shock, the synthesis of normal proteins has resumed. In pupae, the time course is similar after the milder shock. However, the more severe shock causes a drastic reduction in total protein synthesis for at least one hour after the shock. It has been shown previously that this shock causes the production in pupae of stage specific phenocopies at high penetrance, an effect which is not observed after a shock at 37.5°C for 20 minutes. The drastic decrease in total protein synthesis and the subsequent occurrence of anomalies in the resumed program of gene expression and their possible relation to phenocopy production are discussed.

The increase in activity of phenol oxidase after a shock of 40°C for 40 minutes has been investigated to determine whether a component of the enzyme is a heat shock protein. This hypothesis was tested by measuring the extent of co-banding of ^{35}S-methionine labeled proteins from heat shocked and non-heat shocked cells with partially purified phenol oxidase in a sucrose gradient. The results do not support such a role for any heat shock protein; a mechanism whereby phenol oxidase activity could increase after heat shock is discussed.

The 84,000 dalton heat shock protein has been purified by ammonium sulfate fractionation, chromatography on hydroxylapatite, and one- or two-dimensional

gel electrophoresis. This purified protein has been used to produce antibodies in rabbits. The antibodies have been used to show, by indirect immunoprecipitation experiments, that this heat shock protein is normally synthesized in at least three D. melanogaster tissues. Indirect immunofluorescence experiments using these antibodies indicate that the 84,000 dalton protein is also present on the chromosomes of normal and heat shocked salivary glands at the interband regions. Evidence is presented to show that this binding pattern may simply reflect the high concentration of this protein in the cell.

In a separate investigation, the effects of heat shock on the protein synthesis pattern in HeLa cells have also been analyzed.

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General Introduction

Major regions of gene activity are easily visualized on the polytene chromosomes of certain cells in Diptera. The regions of intense transcriptional activity form "puffs" (1, 2). Moreover, purified radioactively-labeled RNA can be hybridized in situ to the chromosomes, and sites of hybridization, detected by autoradiography (3). Thus, species possessing polytene chromosomes offer a unique system for analyzing gene activity and for mapping genes.

Several treatments of intact Drosophila or of excised salivary glands from Drosophila have been shown to alter radically the puffing activity in the salivary gland nuclei. The treatments include exposure to heat (4-6), to cold (1), to uncouplers of oxidative phosphorylation (4, 7), and to hormones (8, 9). Ritossa (4, 7) first noticed that exposure of D. busckii larvae, normally kept at 25°C, to heat (30°C) for 30 min or to uncouplers of oxidative phosphorylation induces puffing at a limited and reproducible number of sites in the chromosomes. Moreover, the same loci puff in response to both treatments. D. melanogaster also has the same property of puffing at specific loci in response to heat and to uncouplers of oxidative phosphorylation (5) but the puff loci are different from those in D. busckii. The regions of heat-induced puffs also puff during recovery from anoxia (7, 10, 11).

Ashburner, who had already made a detailed analysis of regular changes in puffing activity associated with development in D. melanogaster (12, 13), carried out a careful study of the puffing response to heat. In particular, he investigated the effects of exposure to different elevated temperatures and of exposure at high temperature for different lengths of time (11). In D. melanogaster he found

that the appearance of puffs at regions 33B, 63BC, 64F, 67B, 70A, 87A, 87B, 93D, 95D, and occasionally 48E is accompanied by the regression of most preexisting puffs. Five of the heat-induced puff loci, 33B, 67B, 87A, 93D, and 95D, have been observed to puff normally in third instar larvae and/or prepupae. Another, 63BC, is puffed in 7 day lethal giant larvae l(2)gl. These larvae never form pupae, and are suspected to be deficient in a hormone secreted by the ring gland (14). This same region forms a puff in isolated salivary gland nuclei incubated in high concentrations of sodium (cited in 11), and in salivary glands cultured in the abdomens of adult flies (11).

That these heat shock puffs do in fact represent a change in gene expression was established by Tissières _et al._ (15), who showed that the heat shock puff regions were sites of uridine incorporation, and that the set of translation products (polypeptides) after heat shock was radically different from the set of products synthesized before heat shock. Larval and prepupal salivary glands, as well as larval brains, Malpighian tubes, and adult brains, synthesize at least eight new polypeptides after heat shock (15). The molecular weights, as determined by Mirault _et al._ (16) are as follows: 84,000, 70,000, 68,000, 34,000, 27,000, 26,000, 23,000, and 22,000 daltons. At the same time, the normally synthesized proteins are synthesized at a much reduced rate. _D. melanogaster_ synthesizes the same heat shock proteins during recovery from 2 hr under nitrogen (17).

It has been inferred that this heat shock response is general; that is, heat shock protein synthesis is induced in all tissues at all developmental stages. So far only a minor exception has been brought forward (17). Indeed, _D. melanogaster_ tissue culture cells also synthesize the eight major heat shock proteins after heat treatment (18).

D. hydei has also been tested for heat shock protein synthesis and has been reported to synthesize only six heat shock proteins, of molecular weights 70,000, 67,000, 38,000, 26,000, 25,000, and 20,000 (17, 19). Apparently proteins corresponding to *D. melanogaster's* 84,000 dalton and 23,000 or 22,000 dalton heat shock proteins are not synthesized. It seems possible, though, that two lower molecular weight bands have not been resolved on the gels in the work cited above, as is often the case with the 23,000 and 22,000 dalton proteins in *D. melanogaster*. *D. hydei* salivary glands synthesize the same set of proteins during recovery from exposure for 2 hr to nitrogen, during recovery from incubation in the presence of 2,4-dinitrophenol or vitamin B-6, and during incubation in the presence of sodium arsenite (19). In the last case, synthesis of an extra protein is induced; this protein migrates slower than the 70,000 dalton protein and may be the equivalent of the *D. melanogaster* 84,000 dalton protein.

In *D. melanogaster* tissue culture cells raised to 37°C, the preexisting polysomes disaggregate within 10 min (18). By 30 min after the onset of the temperature shock, new, larger polysomes, which synthesize primarily the heat shock proteins, have appeared. Mirault *et al.* (16) have reported that at least some preexisting messenger RNAs remain intact in the cytoplasm of heat treated cells.

Radioactively labeled poly(A)-containing and polysomal poly(A)-containing RNA have been isolated from heat shocked *D. melanogaster* tissue culture cells (18, 20). When this RNA is hybridized *in situ* to polytene chromosomes, the 87BC region* is heavily labeled (18, 20), but most of the other heat shock puff regions also show hybridization. Region 64F is not labeled (20). Region 85D, not puffed

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*The major heat shock puff was first assigned to region 87B (5, 11). Subsequently it was reassigned to region 87C (18).

after heat shock in salivary glands, hybridizes substantially with tissue culture heat shock poly(A)-containing RNA (20).

Polysomal poly(A)-containing RNAs from heat treated tissue culture cells can be separated into two size classes on a sucrose gradient (16, 21). The RNAs in fractions from such a gradient have been translated in vitro (16, 21) or hybridized to the chromosomes in situ (21). Thus, approximate map assignments of the coding regions for the heat shock proteins have been made (21). The assignments are as follows: the 84,000 dalton protein to 63BC, the 70,000 dalton protein to 87A and/or 87BC, the 68,000 dalton protein to 95D, and the 23,000 and/or 26,000 dalton proteins to 67B.

Further mapping data for the 70,000 dalton protein have come from genetic and cloned DNA experiments. Ish-Horowicz et al. (22) have shown that animals homozygous for a deletion on the right arm of chromosome 3 which includes 87A and 87C fail to synthesize the 70,000 dalton protein after heat shock, whereas animals homozygous for a deletion of 87C (not including 87A) do synthesize that protein after heat shock.

A library of cloned genomic D. melanogaster DNA has been screened for hybridization to polysomal poly(A)-containing RNA from heat shocked tissue culture cells (23). Two cloned sequences which hybridize in situ to 87A and 87BC were found. By hybridization-arrested translation using either of these two cloned DNAs, it was determined that both of these recombinant DNAs contain coding sequences for the 70,000 dalton protein, although the coding and adjacent sequences in the two recombinant DNAs are different.

Petersen, Moller, and Mitchell (personal communication) have obtained genetic evidence for the coding of the 27,000 dalton proteins and the 23,000 dalton

protein at map region 67B. As they have seen by gel analysis of ^{35}S-methionine labeled proteins from adult ovaries, homozygous flies synthesize two species of "27,000 dalton" heat shock proteins, and different stocks of wild-type D. melanogaster synthesize different electrophoretic variants of the 27,000 dalton proteins. They have also found flies that synthesize an electrophoretic variant of the 23,000 dalton heat shock protein. Thus, by carrying out crosses of these flies which synthesize electrophoretic variants with flies bearing recessive visible markers, they have been able to map all three proteins.

Other RNAs, besides those translated into heat shock proteins, are transcribed in tissue culture cells exposed to 37°C. As was mentioned above, RNA hybridizable to 85D, a region not puffed in chromosomes of heat treated salivary glands, is synthesized in heat treated tissue culture cells. Histone RNAs continue to be transcribed (24) and translated (16). tRNAs, the precursor of 5S RNA and at least two other low molecular weight RNAs continue to be labeled after heat shock (25, 26). The accumulation of the $5S^{+}$ RNA in heat shocked cells (25, 26) brings to attention another effect of heat shock, failure to process normally synthesized RNA correctly. Lengyel and Pardue (27) have shown that the 38S ribosomal RNA precursor also accumulates in heat shocked cells. Ellgaard and Clever (28) have also shown that processing of the ribosomal RNA precursor is stopped in salivary glands exposed to 37°C. The severity of the heat shock may dictate how much normal RNA synthesis persists during or after heat treatment.

Lis et al. (29) have isolated from a D. melanogaster recombinant DNA library three clones containing overlapping sequences from region 87BC. The clones were selected by hybridization to polysomal poly(A)-containing RNA from heat shocked tissue culture cells. The RNA species that hybridize to these

sequences have not yet been associated with any particular translation products. Lis et al. (29) have suggested that this RNA may serve a regulatory role or that it may be a by-product of primary transcript processing.

No physiological role for any of the heat shock proteins has been established; however, it has been suggested that some of the heat shock proteins are mitochondrial enzymes involved in respiratory function (30-32). The basis for the suggestion is the fact that most of the heat shock puff inducing treatments affect respiratory metabolism in some way; for example, after heat or anoxia treatment, oxygen consumption is stimulated, and consumption of substrates of a variety of mitochondrial enzymes is increased (33, 34). Other conditions that affect respiratory metabolism and induce heat shock puffs are treatments with dinitrophenol, sodium salicylate, sodium azide, dicumarol, which are uncouplers of oxidative phosphorylation, and antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide, which inhibit electron transport (for reviews see 31, 32). Circumstantial evidence has been presented for an association of NADH dehydrogenase activity and isocitrate dehydrogenase activity with heat shock proteins (35, 36).

Two studies have been made which may open the way for determining the molecular events which directly trigger heat shock puffing. Compton and McCarthy (37) have succeeded in inducing heat shock puffs in D. melanogaster nuclei from cells kept at 25°C by incubating the nuclei in cytoplasm from heat-treated cells. Sin (38) has found that a wash of heat-treated mitochondria, when injected into salivary gland cells of D. hydei, could induce puffing at at least two heat shock loci. The effective substance(s) in the mitochondrial wash is heat labile and nondialyzable (38).

In this thesis are described studies on the effect of heat shock on protein synthesis. The duration of heat shock protein synthesis after a heat shock of 37.5°C for 20 min or a heat shock of 40.2°C for 40 min has been determined for flies in the prepupal and pupal stages, respectively. Also, the resumption of the synthesis of normal proteins has been analyzed in detail in pupae recovering from a 40.2°C, 40 min heat shock. The synthesis of the heat shock proteins on cytoplasmic rather than mitochondrial ribosomes has been shown. The suggestion that a phenol oxidase activity is associated with the heat shock proteins has been investigated. One of the heat shock proteins has been purified and antiserum against it produced. This antiserum, or its gamma globulin fraction has been used to determine whether this protein is normally synthesized in cells kept at 25°C, and whether the protein binds to any specific sites on the polytene chromosomes.

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CHAPTER 1a

KINETICS OF HEAT SHOCK PROTEIN SYNTHESIS

Introduction

Information about the induction and duration of heat shock protein synthesis is necessary for studies that may involve purification of any of the heat shock proteins. This information might also prove useful for any future studies aimed at investigating the mechanisms involved in the changing translational specificity after heat shock.

Salivary glands from prepupae heat shocked at about 7.5 hr after puparium formation were used to study the kinetics of heat shock protein synthesis. The larval salivary glands are one of the few tissues that persist to pupation (12 hr after puparium formation), and they are easily dissected. Previous studies have determined the conditions under which, in a 20 min pulse, six glands take up and incorporate ^{35}S-methionine into protein at a rate sufficient to give good autoradiograph exposures within a week (1; A. Tissières and H. K. Mitchell, personal communication).

Materials and Methods

Fly stock and culture

The Oregon R wild-type stock of Drosophila melanogaster was cultured in mass quantities and selected for synchrony by flotation in water as described by Mitchell and Mitchell (2). The prepupae varied within 30 min to 1 hr of the average age stated in the text, depending on the flotation intervals.

Heat shock

Prepupae were transferred to a 25 x 95 mm vial containing a small piece of moist filter paper. The vial was stoppered with a sponge cylinder, and immersed in a water bath set to the heat shock temperature, so that the prepupae were well below the level of the water.

Labeling

Six salivary glands were dissected at various ages or at various times after heat shock in "A" medium (3). Usually dissection of 6 glands was accomplished in less than 10 min. "Time after heat shock" denotes the elapsed time between the end of the heat shock and the beginning of the labeling pulse. Labeling was carried out essentially as described by Tissières et al. (1). The six glands were incubated for 20 min at room temperature in about 4 μl of "A" medium containing 10-20 μCi of ^{35}S-methionine (200-400 Ci/mMole, New England Nuclear). Incorporation was stopped by the addition of cold 10% TCA. The tissue was washed first with 95% ethanol, then with a 1:1 mixture of chloroform and methanol. The tissue was dried and dissolved in the SDS sample buffer described by Tissières et al. (1).

Gel electrophoresis and autoradiography

Electrophoresis and autoradiography were carried out as described by Tissières et al. (1). Polyacrylamide gels were made up in SDS-Tris buffer (4). The samples were run at 4°C on a 12.5% acrylamide gel (13.5 x 9 cm) with a 7.5% acrylamide stacking gel, at 10-12 ma for 4-6 hr. The gels were stained (5), dried

(6) and placed in contact with Kodak SB-5 no screen X-ray film to expose for autoradiography.

Measurement of band intensities

Autoradiograms were scanned on a Joyce Loebl densitometer and areas were measured using a programmed Hewlett Packard desk computer coupled to a digitizer (courtesy of N. Davidson).

Results and Discussion

Table 1 shows that the threshold of induction of heat shock protein synthesis is between 2 and 6 min at 32°C and less than 1 min at 37.5°C. At temperatures above 37.5°C, heat shock protein synthesis was induced but with increasing temperatures and durations of heat shock, the rate of labeling of proteins decreased (data not shown).

The viability of the flies after heat treatment is an important consideration if biochemical experiments after heat treatment are to be carried out. The animals show good viability after 20 min at 37.5°C. Longer treatments at this temperature decrease the viability, so that larvae kept 4 hr at 37.5°C have 70-85% mortality (3). A shock of 25 to 39°C for 1 hr is 100% lethal to larvae (3).

A heat shock of 37.5°C for 20 min was used initially in this laboratory for studies on protein synthesis (1) because heat shock puffing in larvae maintained at this temperature lasts longer than at the other temperatures tried (3) and, after 20 min at 37.5°C, puffing is at or near maximum intensity (3). Furthermore, heat shock puffs are smaller throughout in larvae kept at 39°C, the next higher

Table 1. Induction of Heat Shock Protein Synthesis

Temperature of heat shock (°C)	Duration of heat shock	Elapsed time from end of shock to beginning of labeling pulse (min)	Duration of labeling pulse (min)	Average age of prepupae at time of heat shock (hr)*	Synthesis of 70K dalton heat shock protein**	Synthesis of at least the 84K, 70K, and 68K dalton heat shock proteins**
32	63 sec	10	20	8.5	ǂ	no
32	2 min	20	20	6.6	ǂ	no
32	2 min	10	20	7.5	no	no
32	6 min	<15	20	7.3	yes	yes
32	10 min	10	20	8.0	yes	yes
32	21 min		20	7.3	yes	yes
37.5	1 min	<15	6	7.8	yes	yes
37.5	1 min	17	20	8.5	yes	yes
37.6	1 min	8	20	7.8	yes	yes
37.5	20 min	8	20	8.0	yes	yes
38.5	6 min	17	20	7.3	yes	yes
38.5	15 min	11	20	7.8	yes	yes
38.5	20 min	9	20	7.3	yes	yes
39.0	5 min	10	20	8.0	yes	yes
39.0	11 min	11	20	8.0	yes	yes

*Age is given as hours after puparium formation.

**Determined by autoradiography of SDS gels as described in Materials and Methods.

ǂIt was difficult to tell if the synthesis of a 70K dalton protein was induced at a low level, since glands of prepupae at this age normally synthesize a protein of about the same mobility.

temperature used in the above cited puffing studies (3). Since a 37.5°C heat shock for 20 min seemed likely to induce the longest lasting expression of heat shock genes in Drosophila cells, this condition was used to follow the kinetics of heat shock protein synthesis.

Figure 1 shows the autoradiogram of salivary gland proteins labeled at four different times after heat shock. For comparison, the protein synthesis patterns of non-heat shocked controls of similar ages are also shown. Labeling of the heat shock proteins is strongest immediately after the heat shock (Fig. 1b) and by 3 hr after the heat shock (Fig. 1h) has decreased to undetectable levels. Figure 2 shows the densitometer scans of the autoradiogram shown in Figure 1. The scan in Figure 2b does not resolve well the two bands representing the 70,000 and 68,000 dalton heat shock proteins, but in Figure 2d the two bands are resolved as a double pointed peak.

The labeling of the 70,000 and 68,000 dalton proteins decreases faster than does the labeling of the 23,000 and 22,000 dalton heat shock proteins. Based on integrations of the scans shown in Figure 2, and of scans from other experiments, 28% of the incorporated ^{35}S-methionine is in the 70,000 and 68,000 proteins 10 min after heat shock and 2 hr later, these proteins represent only 6% of the label (Fig. 3). In contrast, by similar measurements, less than 5% of the label is in the 22,000 and 23,000 dalton proteins 10 min after heat shock, but at 2 hr after the shock, 3% of the label is still found in these proteins. These data suggest a differential regulation of the synthesis of the individual heat shock proteins.

The sample labeled 3 hr after heat shock (age 11 hr) (Figs. 1h, 2h) shows synthesis of a high molecular weight protein which was being synthesized at the time of heat shock (Figs. 1a, 2a). However, the 10.5 hr control sample (Figs. 1g,

Figure 1. Autoradiograph of an SDS gel on which samples labeled at various times after the end of a 20 min heat shock at 37.5°C were run. Prepupae were heat shocked at 7.5 hr after puparium formation.

(a, c, e, g) prepupae maintained at 25°C until dissection was performed at the following ages: (a) 7.5 hr, (c) 8.5 hr, (e) 9.5 hr, (g) 10.5 hr.

(b, d, f, h) prepupae heat shocked at 7.5 hr and then kept at 25°C until dissection was performed at the following ages (intervals after heat shock [hs]): (b) 8 hr (hs + 8 min), (d) 9 hr (hs + 1 hr, 15 min), (f) 10 hr (hs + 2 hr, 16 min), (h) 11 hr (hs + 3 hr, 12 min).

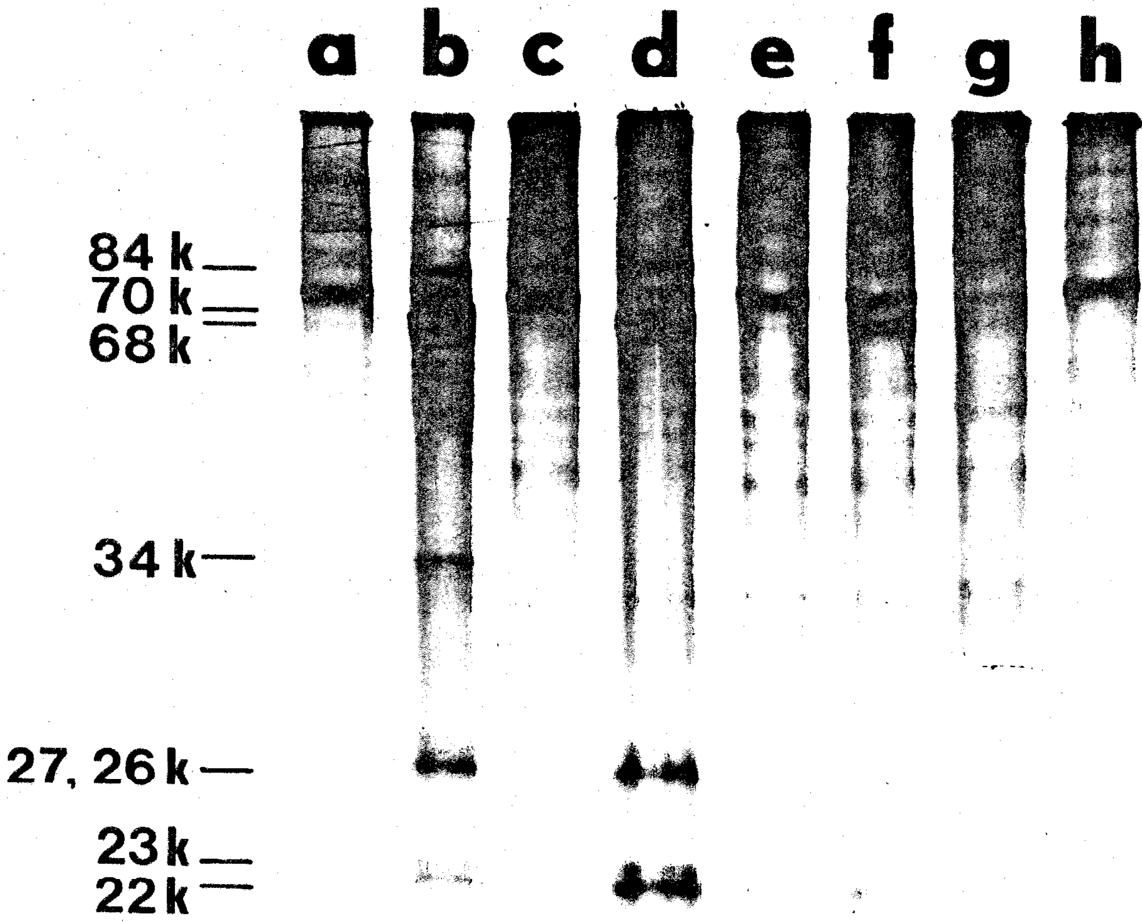

a
b
c
d
e
f
g
h
84 k
70 k
68 k
34 k
27, 26 k
23 k
22 k

Figure 2. Densitometric scans of an autoradiogram of the same gel shown in Figure 1. Samples are labeled as in Figure 1, each panel in Figure 2 corresponding to the respectively lettered lane in Figure 1. The panels on the left represent non-heat shocked controls, the panels on the right represent heat shocked samples. The arrow in each panel on the right points to the position of migration of the 70,000 dalton heat shock protein. Migration through the gel is from left to right.

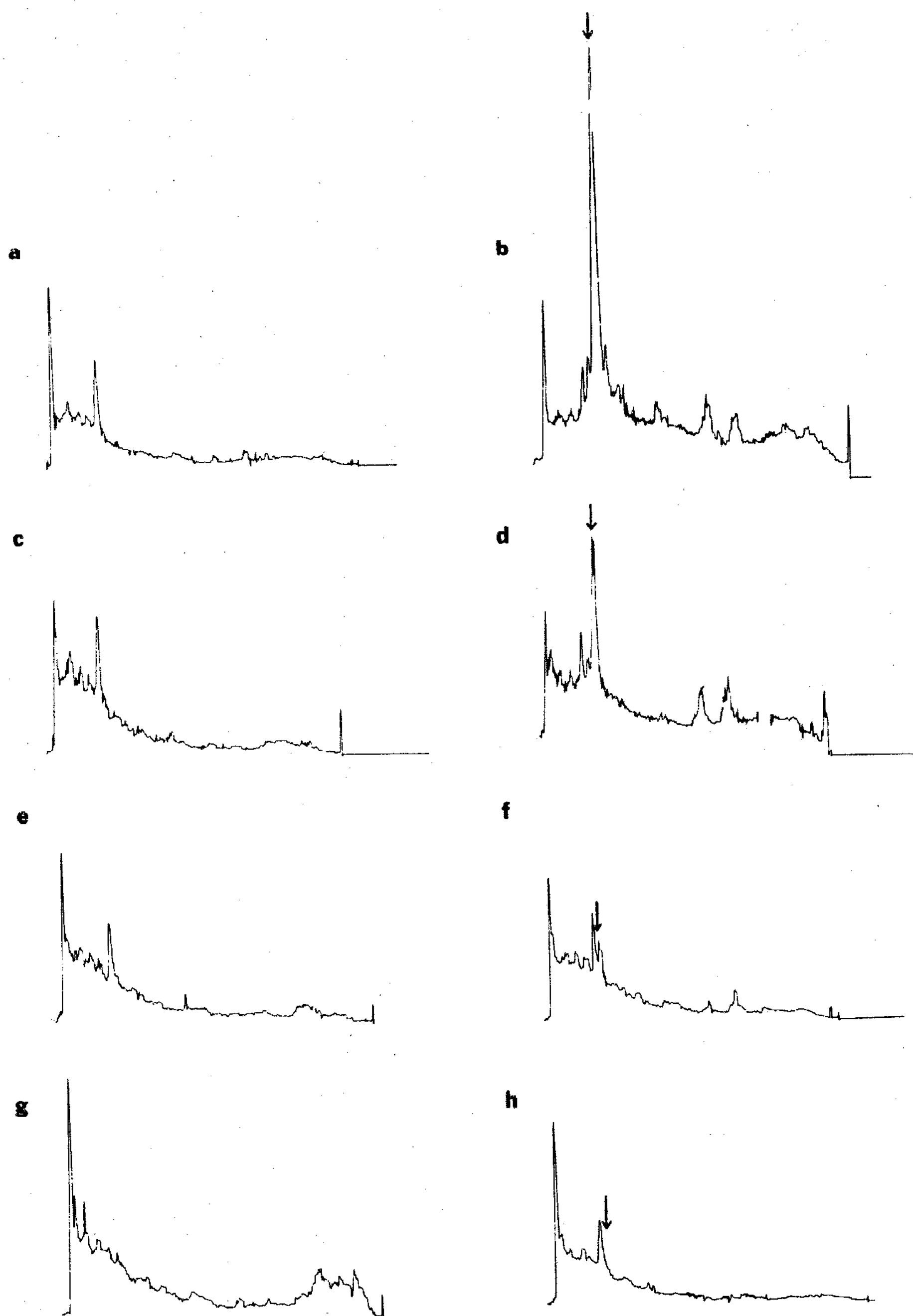
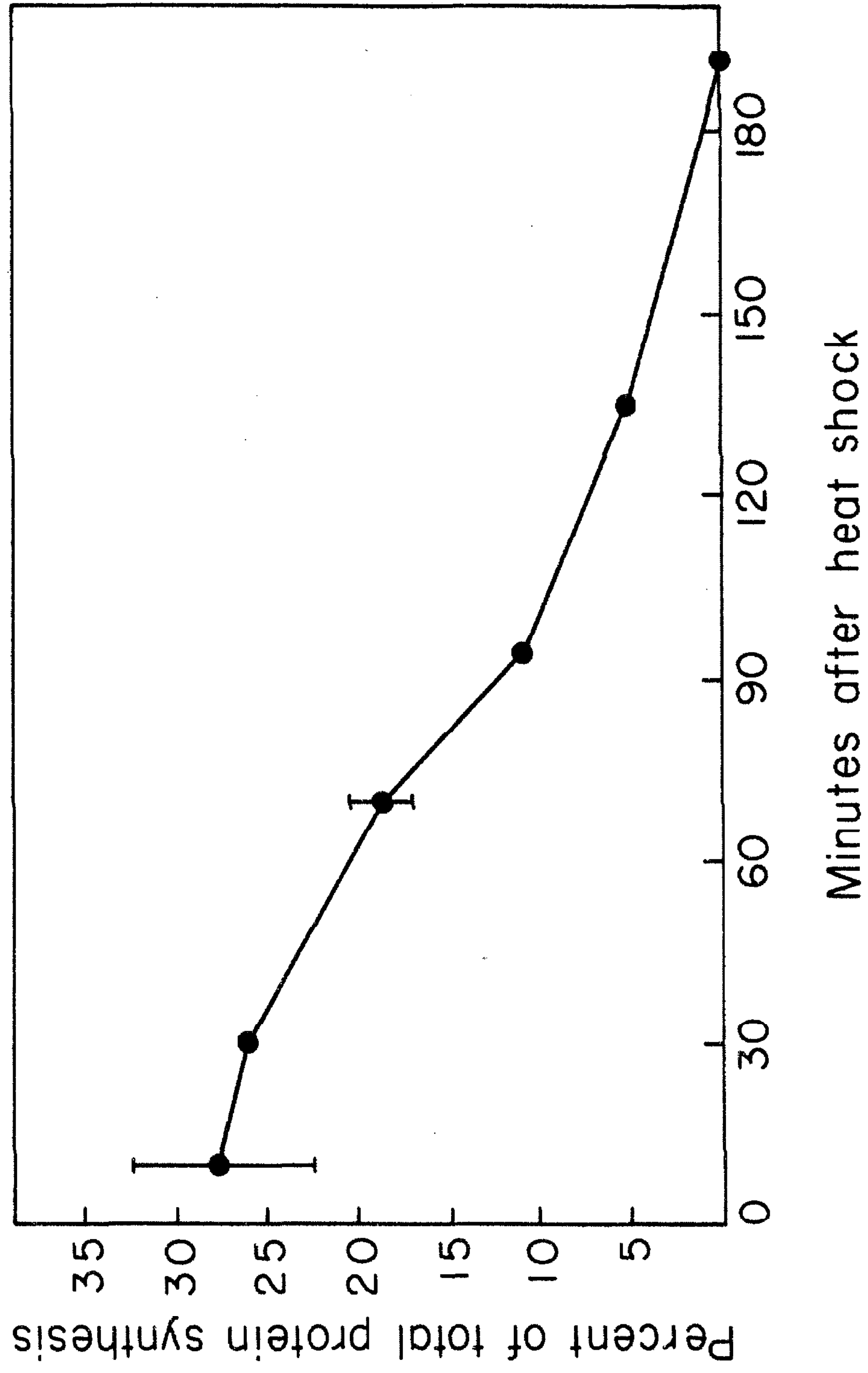

a
b
c
d
e
f
g
h

Figure 3. Labeling of the 70,000 and 68,000 dalton heat shock proteins as a percentage of the total ^{35}S-methionine incorporated into salivary gland proteins at various times after a 20 min heat shock at 37.5°C. The labeling of pairs of heat shock proteins is measured as a percentage of total incorporation rather than as an absolute number of counts per minute because the sample size, 6 glands, is so small that variability due to cell size and individual genetic variation is large. The areas under the total curve and under the peak(s) representing the 70,000 and 68,000 dalton heat shock proteins were measured on densitometer scans of autoradiograms of three different slab gels, including the gel shown in Figure 1. Samples for all three gels were labeled and prepared as described in Materials and Methods and in the legend to Figure 1. Two of the time points, ±5 min, were held in common by two or three of the gels, and for these, the average percentages and the standard deviations are indicated. At 3 hr after heat shock, the 70,000 dalton band is no longer detectable on the autoradiogram, and therefore its contribution to the total area could no longer be measured.


Percent of total protein synthesis
35
30
25
20
15
10
5
0
30
60
90
120
150
180
Minutes after heat shock

2g) is no longer synthesizing this protein. The heat shock apparently interrupts normal protein synthesis, which resumes as the synthesis of the heat shock proteins decreases.

Other information on the kinetics of heat shock protein synthesis has been published since these experiments were performed. Lewis et al. (7) have shown that heat shock protein synthesis lasts for at least 1 hr but not as long as 4 hr after a 20 min shock at 37°C to late third instar larvae. Mirault et al. (8) have reported that D. melanogaster tissue culture cells at 37°C continue to synthesize heat shock proteins, but after 2 hr at 37°C, synthesize them at a gradually decreasing rate. By 6 to 8 hr after the onset of the temperature treatment, these proteins are synthesized at only 50% of the initial rate. Moreover, it is now known that the heat shock proteins do not turn over for at least 2 hr in salivary glands cultured in vitro at 25°C after exposure to 37°C for 20 min (7); nor do these proteins turn over in tissue culture cells for at least 20 hr after 1 hr exposure to 37°C (8).

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CHAPTER 1b

PATTERNS OF PROTEIN SYNTHESIS FOLLOWING HEAT SHOCK IN PUPAE

Introduction

A heat shock of 37.5°C for 20 min induces the synthesis of eight new proteins in larval salivary glands of *Drosophila melanogaster* while reducing the preexisting synthesis of normal proteins (1, 2). The synthesis of this new set of proteins with concomitant suppression of normal protein synthesis is a rather general response to heat shock: these heat shock proteins are synthesized in all the other tissues that have been investigated; namely, in larval Malpighian tubules, midgut, brain, and imaginal discs, and in adult brain, ovaries, testes, Malpighian tubules, midgut, and abdominal epithelium (1-3). McKenzie *et al.* (4) found that these heat shock proteins were synthesized in *Drosophila melanogaster* tissue culture cells as well, after exposure of the cells to 37°C.

Results from *in situ* hybridization experiments between *in vivo* labeled RNA from heat shocked cells and polytene chromosomes (4, 5) taken together with results from *in vitro* translation experiments using polysomal RNA from heat shocked cells (6, 7) have indicated that these heat shock proteins are the gene products of some of the salivary gland chromosomal puff regions observed in heat shocked larvae by Ritossa (8) and Ashburner (9). The heat shock phenomena in *Drosophila* provide, therefore, an excellent system for studying regulation of gene expression in eukaryotic cells.

To provide more information toward understanding the mechanics of the induction of heat shock protein synthesis and resumption of normal protein synthesis, and also toward understanding the molecular basis of phenocopy

production, we have analyzed the effect of a more severe heat shock on protein synthesis in pupal tissues. Unlike larvae and prepupae, pupae can withstand fairly well heat shock at 40°C for 40 min (10), a treatment which has drastic effects on the pattern of protein synthesis. Moreover, this temperature treatment, but not a heat shock of 37.5°C for 20 min, produces stage-specific phenocopies, many of which are similar to known mutants (10-13). We have analyzed, on high resolution acrylamide gels, the response in protein synthesis patterns of pupae heat shocked at three ages which are sensitive periods for three different bristle phenocopies.

Mitchell and Lipps (12) found that a double heat shock produces either an enhanced single or a double phenocopy depending on the time interval between heat treatments. These results indicate that heat treatment causes a delay, or interruption, in development. Hence the timing of the normal sequential changes in the pattern of protein synthesis after heat treatment was analyzed to determine the delay in biosynthetic events. The effect on protein synthesis after a double heat shock was also investigated to determine whether pupae would respond to the second heat shock in the same way as to the first heat shock.

We have compared the heat shock response in thoracic epithelial tissue and brain. The first tissue derives from imaginal discs and does not begin growth and differentiation until after pupariation. This tissue, probably as a consequence of its complicated developmental program, shows numerous changes in the pattern of protein synthesis, as analyzed on SDS gels (14). The brain, on the other hand, is one of the few larval tissues to survive metamorphosis. Although it shows some morphological changes after pupariation, the pattern of protein synthesis throughout the pupal stages shows relatively few changes (15).

Materials and Methods

Fly culture

Canton S wild-type stock of Drosophila melanogaster were raised in mass culture at 25°C as described by Mitchell and Mitchell (16). At this temperature the life cycle of fly is as follows: eggs hatch 22 hr after egg laying. The larvae undergo two molts and at 120 hr after egg laying, form puparia. For the next 12 hr, or until head eversion, the animals are called prepupae. At the time of head eversion, they become pupae. Eclosion takes place 4 days after puparium formation. Ages of pupae are referred to as hours after puparium formation.

The animals are synchronized by flotation at 5 hr after puparium formation (16). Individuals were examined under the microscope and abnormal or misshapen ones were discarded; pupae older than 48 hr were also selected for synchronous development of pigmentation (eye color, wing color). The pupae used in the experiments were synchronized to ±1 hr of the indicated times. All of the animals in each experiment were taken from boxes for which egg collections had been made on the same day, except where indicated.

Heat shock and labeling

Pupae were placed in vials, to which warm water was added, and the vials kept immersed in a water bath at 40.2°C for 40 min. Time after heat shock is defined as the time elapsed from the end of the heat shock to the beginning of the labeling pulse.

Thoracic epithelial tissue was dissected as previously described (14). Brains were dissected with the thoracic ganglion intact and attached. A sample

of three brains or thoracic tissue from three pupae was labeled in approximately 3 µl of MOPS buffered medium (14) containing about 20 µCi of ^{35}S-methionine of specific activity 570 Ci/mMole (New England Nuclear). After a 25 min incubation at room temperature, about 25°C, samples were washed three times with MOPS buffered medium and transferred to a small chip of glass from a broken siliconized coverslip. The chip was transferred to a small test tube (6 x 50 mm) containing the SDS sample buffer of Fairbanks et al. (17) with the following modifications: the buffer contained 10 mM EDTA, the pH was 7.75, and the DTT was freshly added to the sample buffer for each experiment. Brain samples were dissolved in 30 µl buffer and thoracic epithelial samples, in 40 µl. Samples were kept at room temperature for several hours and then kept frozen at -80°C until a few hours before electrophoresis. Samples were never kept frozen for longer than a week. Just before electrophoresis, the samples were heated at 37°C for 15 min. The entire brain sample or 2/3 of the thorax sample was applied to the gel. Thorax samples treated in this way were no different in their electrophoretic pattern on gels than samples denatured with cold 10% TCA immediately after labeling and washed with 95% ethanol, then with a 1:1 mixture of chloroform and methanol, before dissolution in sample buffer (1).

Gel electrophoresis

Electrophoresis was carried out on slab gels made with an exponential gradient of acrylamide concentration from 10 to 20% (to be described in more detail, 15). The SDS-containing buffers of Laemmli (18) were used. Staining and destaining were carried out essentially as described by Fairbanks et al. (17). The gels were dried and exposed for autoradiography on Kodak SB-5 no screen X-ray

film. Autoradiogram exposures were usually 1 to 2 days long. The autoradiogram in Figure 4b was exposed for a time about 10 times as long.

Phenocopies

Animals from the same heat shocked samples used for the dissections were allowed to develop to adult flies at 25°C and examined for heat-induced phenotypes in the dissecting, phase contrast, or electron microscope (12).

Results

Thoracic epithelial tissue and brain were dissected at various ages (non-heat shocked controls) and at various times after a 40 min exposure of the pupae to 40.2°C; immediately after dissection the tissues were labeled *in situ* with ^{35}S-methionine for 25 min at room temperature (about 25°C). Figures 1, 2, and 3 show the electrophoretic patterns (Coomassie blue staining [1a, 2a, and 3a] and autoradiograms [1b, 2b, and 3b]) obtained when extracts of the labeled tissues were run on 10-20% exponential gradient acrylamide SDS gels. The protein patterns obtained from pupae heat shocked at 34 hr are shown in Figures 1a and 1b, the patterns from pupae heat shocked at 53 hr, in Figures 2a and 2b, and the patterns from pupae heat shocked at 76 hr, in Figures 3a and 3b.

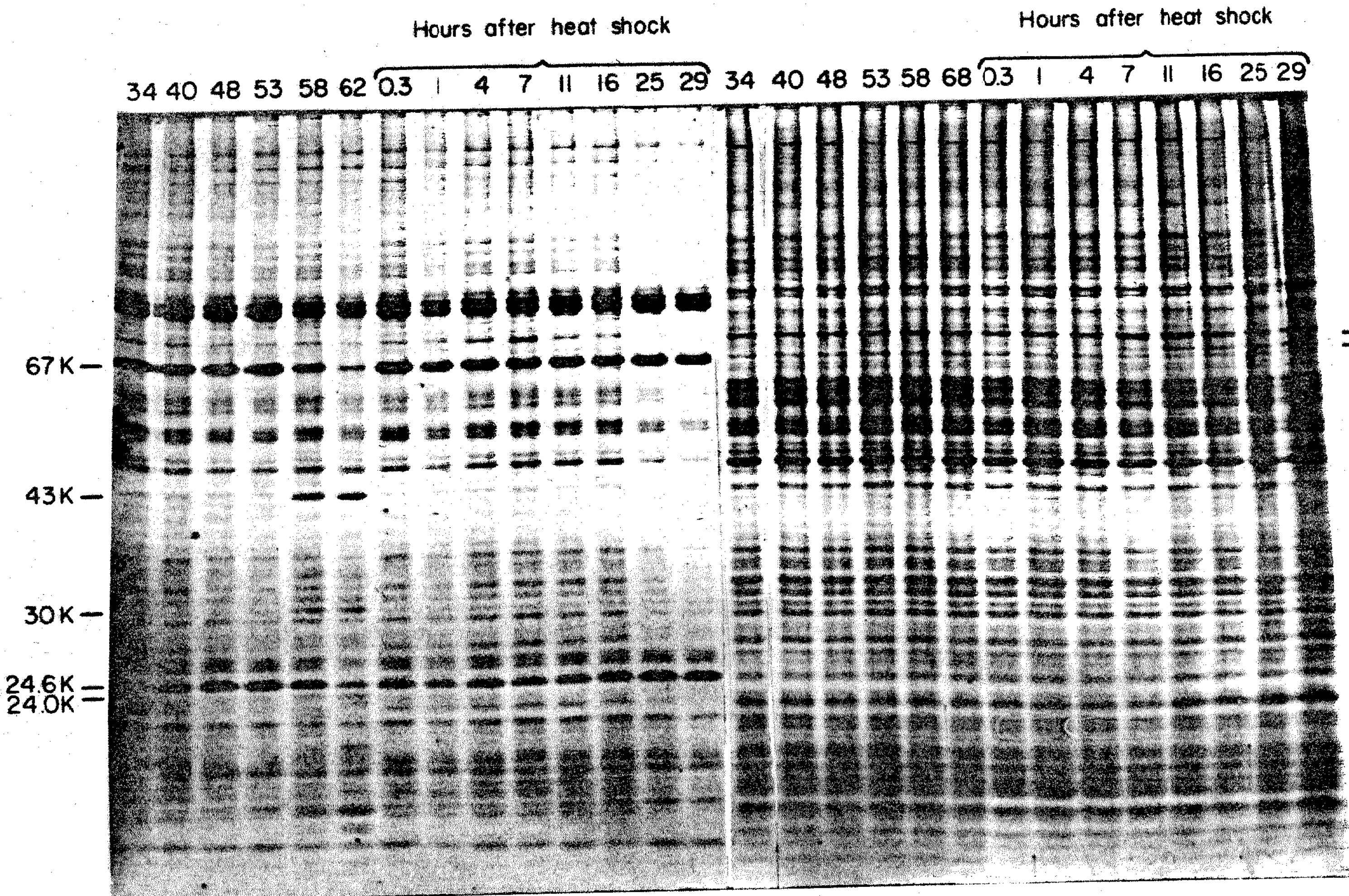
Time course of heat shock protein synthesis

a) Single heat shock

The effects of heat shock at the three different developmental stages and in the two different tissues share some similar characteristics: immediately after heat shock and for at least 1 hr more, the rate of protein synthesis is

Figure 1. Heat shock at 34 hr after puparium formation. (a) Coomassie stained pattern and (b) autoradiogram from 10 to 20% exponential gradient SDS acrylamide gel electrophoresis of ^{35}S-labeled Drosophila melanogaster proteins. Ages given in hours after puparium formation are shown above lanes corresponding to non-heat shocked samples. Samples from animals heat shocked at 40.2°C for 40 min are indicated as hours elapsed after heat shock. Dissection of tissues and pulse-labeling with ^{35}S-methionine was as described in Materials and Methods.

The approximate molecular weights in daltons are indicated along the sides of the gel. In (b) the molecular weights of the heat shock proteins are set off from those of other proteins. The molecular weights of the heat shock proteins were taken from Mirault et al. (6) and a standard curve of molecular weight versus mobility (19) based on these values was used to determine the molecular weights of the other proteins.


34 hrs
a
THORACIC EPITHELIUM
BRAINS
Hours after heat shock
Hours after heat shock
34 40 48 53 58 62 0.3 1 4 7 11 16 25 29
34 40 48 53 58 68 0.3 1 4 7 11 16 25 29
67K
43K
30K
24.6K
24.0K
70K
68K

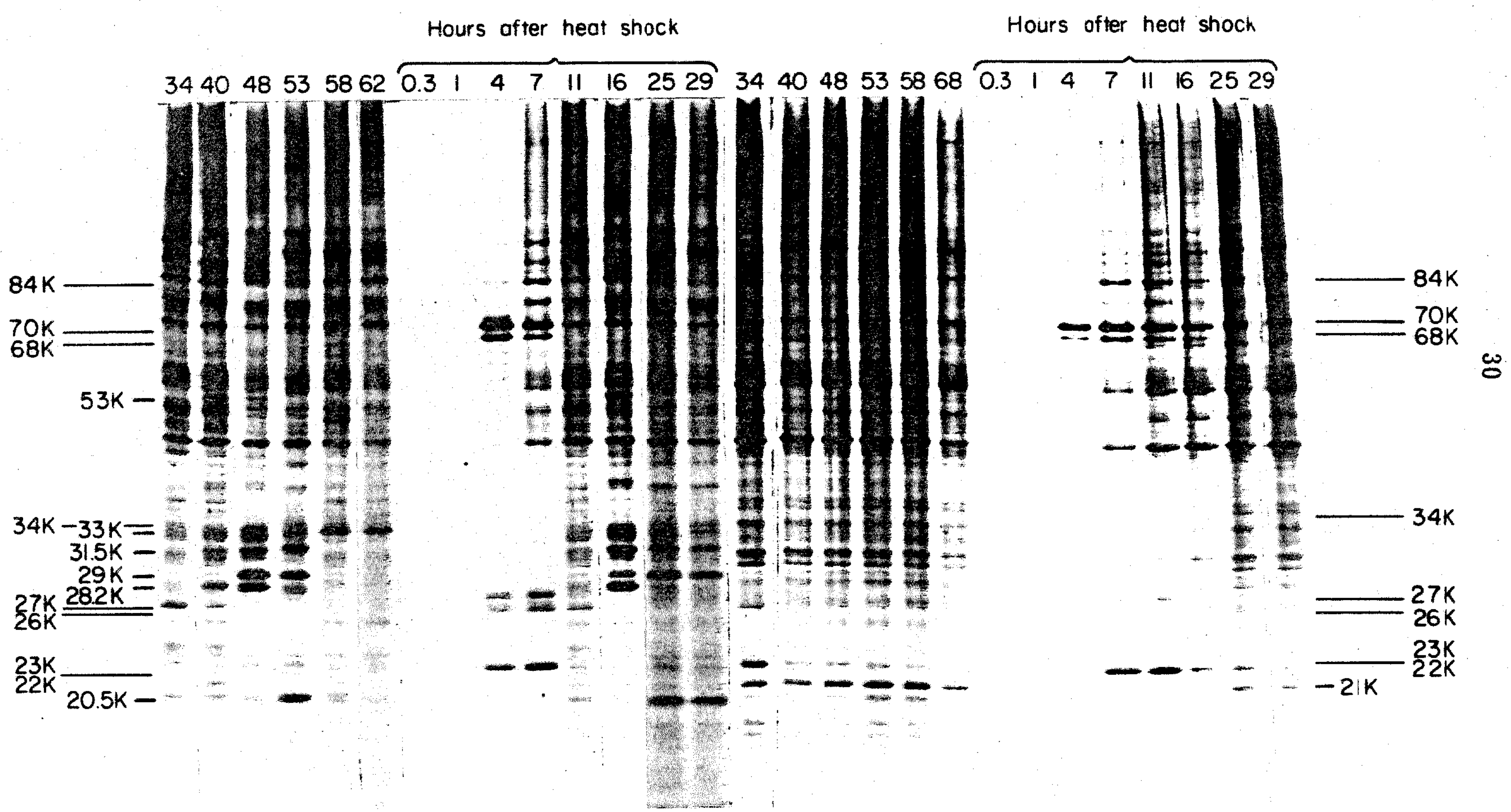
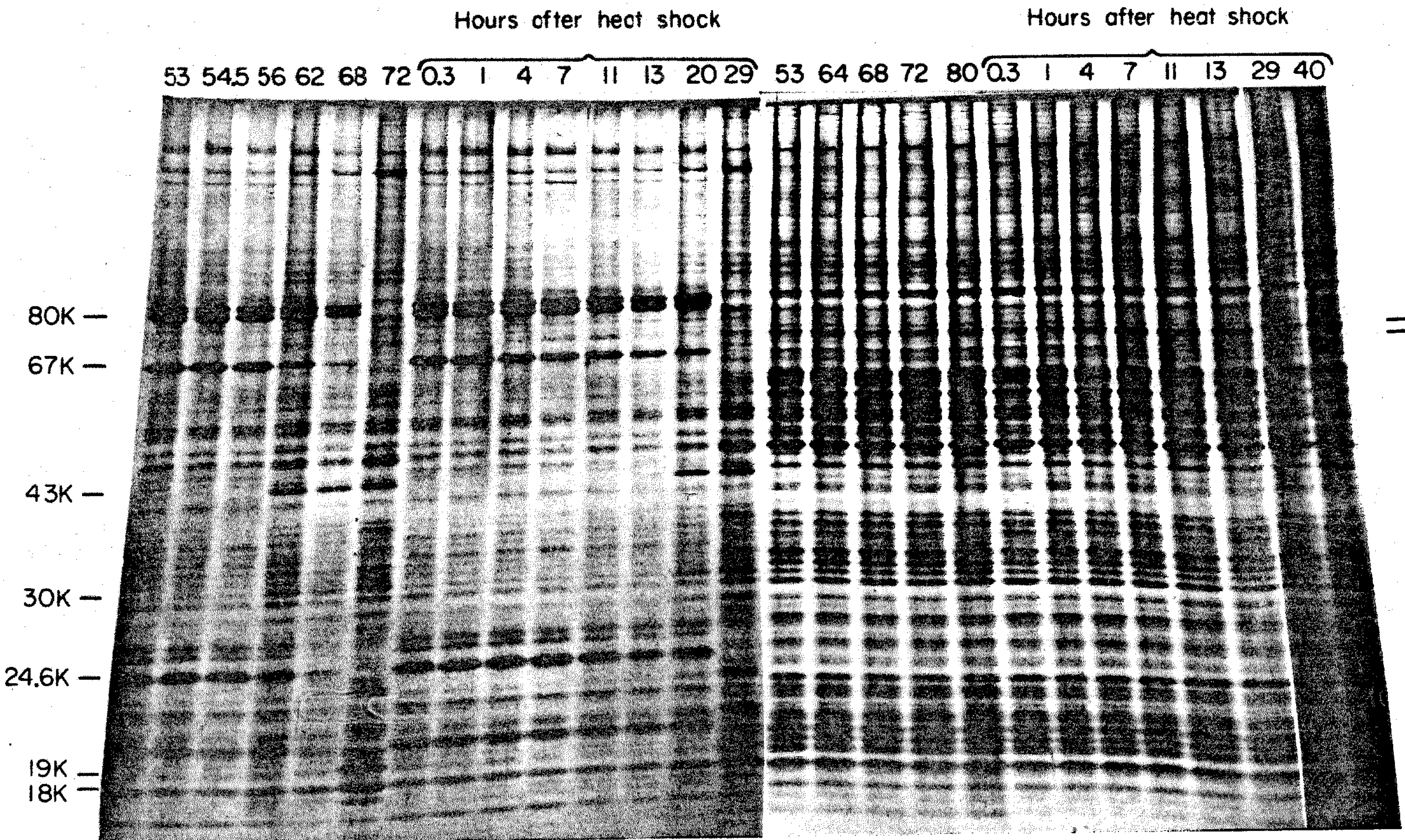

34 hrs
b
THORACIC EPITHELIUM
BRAINS
Hours after heat shock
34 40 48 53 58 62 0.3 1 4 7 11 16 25 29
Hours after heat shock
34 40 48 53 58 68 0.3 1 4 7 11 16 25 29
84K
70K
68K
53K
34K
33K
31.5K
29K
28.2K
27K
26K
23K
22K
20.5K
84K
70K
68K
34K
27K
26K
23K
22K
21K

Figure 2. Heat shock at 53 hr after puparium formation. (a) Coomassie stained pattern and (b) autoradiogram. Electrophoresis was carried out and lanes are marked as described in legend to Figure 1.



53 hrs
a
THORACIC EPITHELIUM
BRAINS
Hours after heat shock
53 54.5 56 62 68 72 0.3 1 4 7 11 13 20 29
Hours after heat shock
53 64 68 72 80 0.3 1 4 7 11 13 29 40
80K
67K
43K
30K
24.6K
19K
18K
70K
68K

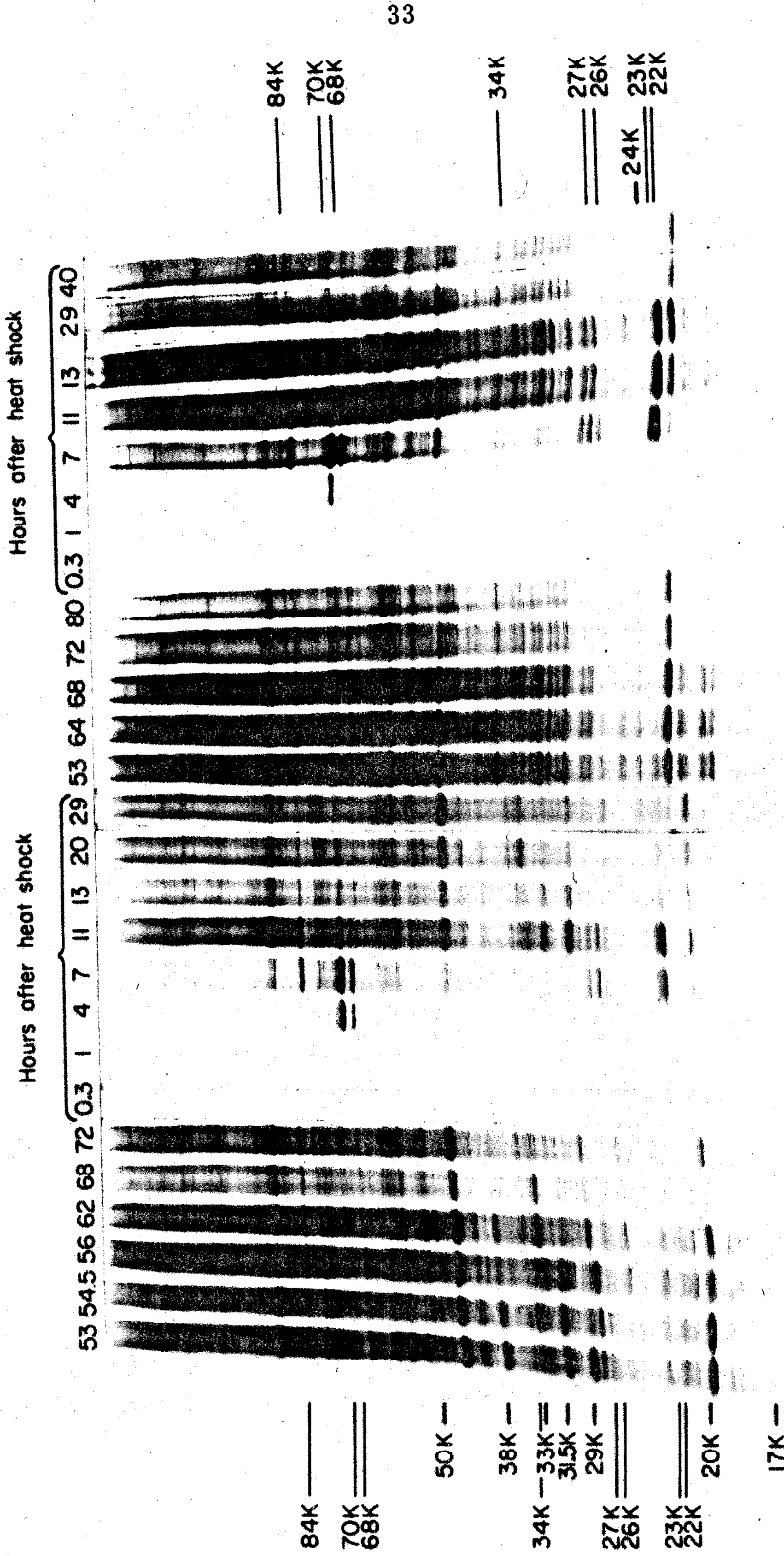
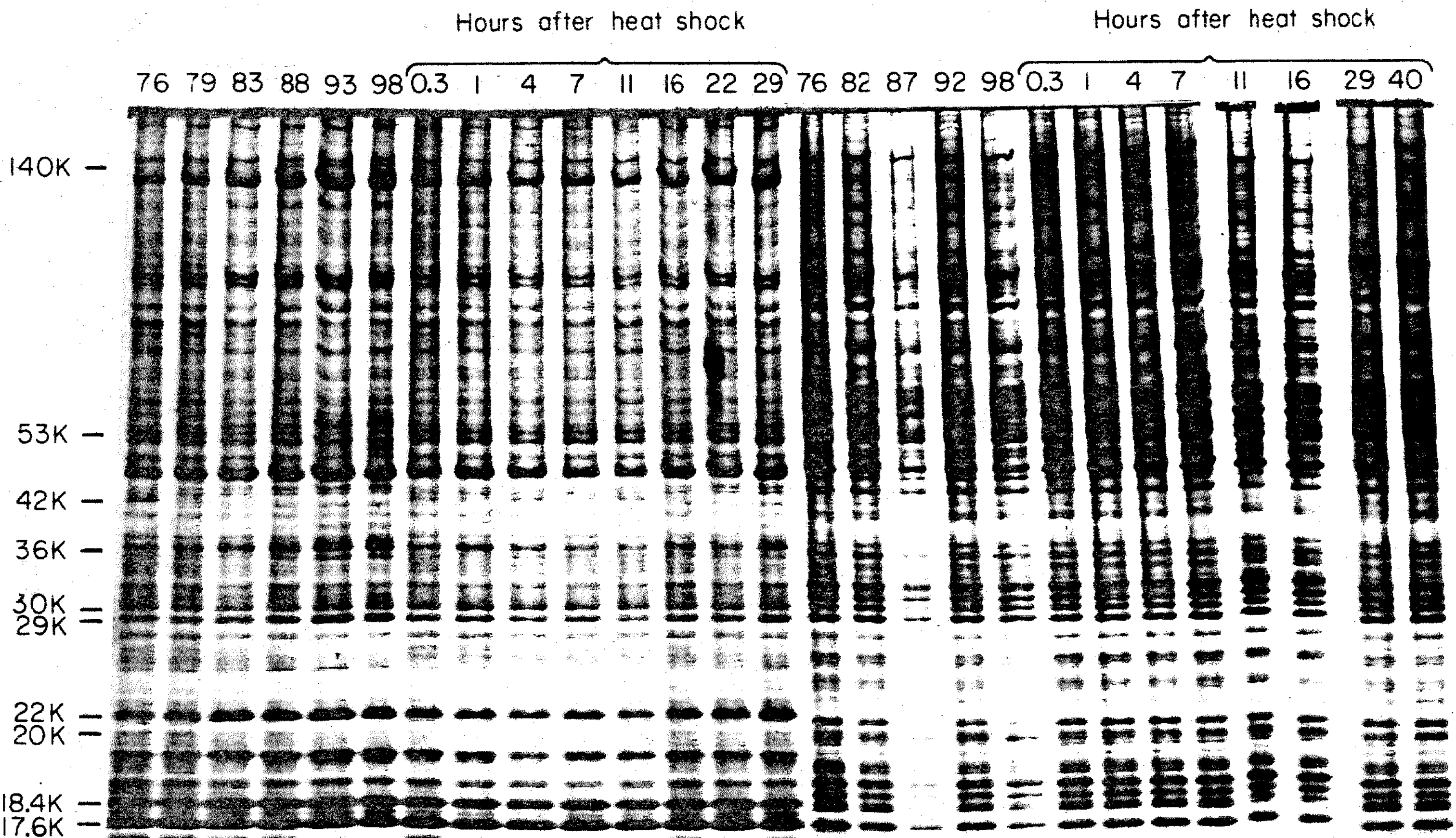
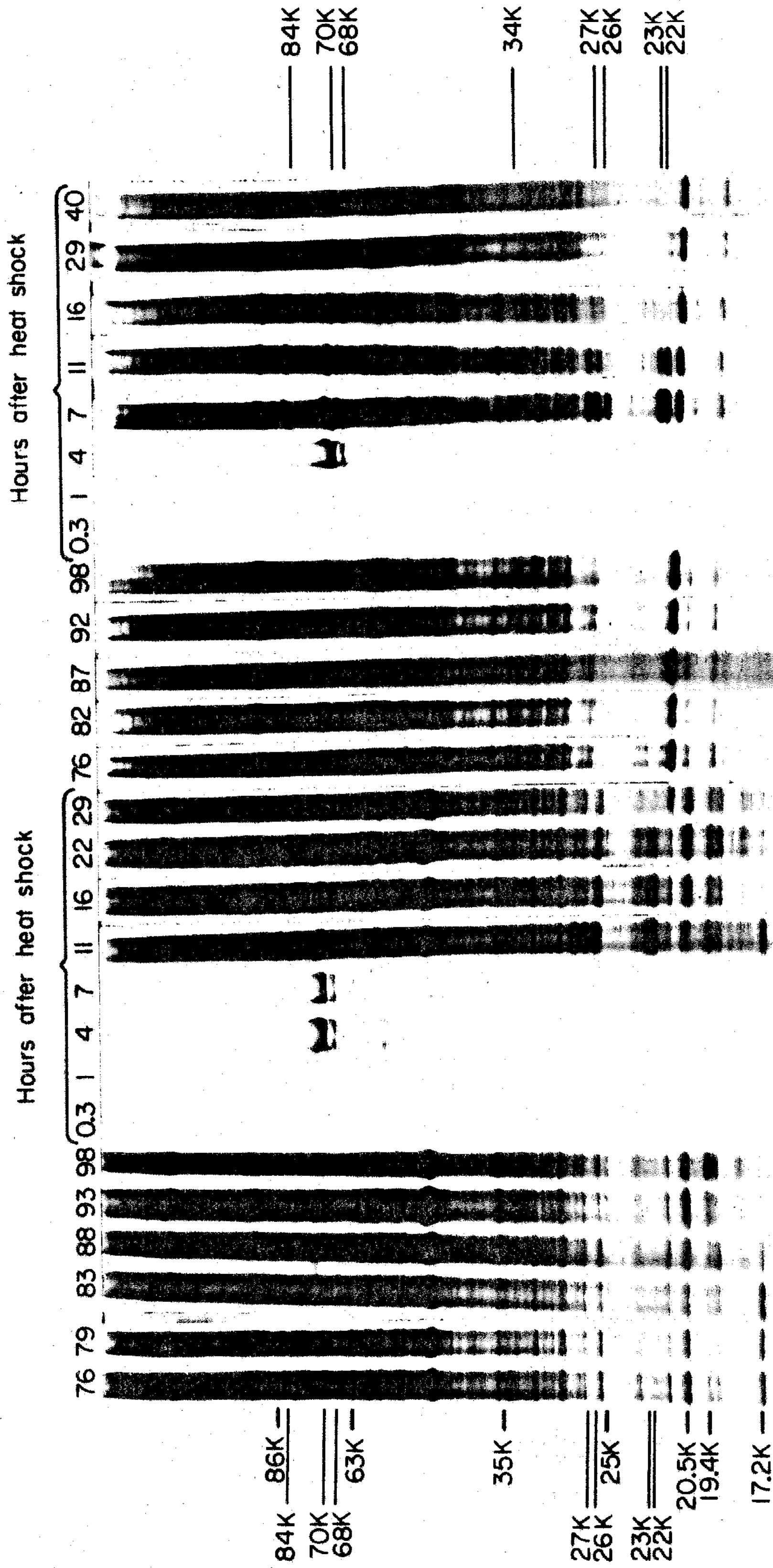

b
53 hrs
THORACIC EPITHELIUM
BRAINS
53 54.5 56 62 68 72
Hours after heat shock
0.3 1 4 7 11 13 20 29
53 64 68 72 80
Hours after heat shock
0.3 1 4 7 11 13 29 40
84K
70K
68K
34K
27K
26K
24K
23K
22K
50K
38K
33K
31.5K
29K
20K
17K

Figure 3. Heat shock at 76 hr after puparium formation. (a) Coomassie stained pattern and (b) autoradiogram. Electrophoresis was carried out and lanes are marked as described in legend to Figure 1. The brain samples taken 11 and 16 hr after heat shock were prepared on a different day from the rest of the samples and were run on a different gel.



76 hrs
a
THORACIC EPITHELIUM
BRAINS
Hours after heat shock
Hours after heat shock
76 79 83 88 93 98 0.3 1 4 7 11 16 22 29
76 82 87 92 98 0.3 1 4 7 11 16 29 40
140K
53K
42K
36K
30K
29K
22K
20K
18.4K
17.6K
70K
68K


b
76 hrs
THORACIC EPITHELIUM
BRAINS
76 79 83 88 93 98
0.3 1 4 7 11 16 22 29
Hours after heat shock
76 82 87 92 98
0.3 1 4 7 11 16 29 40
Hours after heat shock
84K
70K
68K
27K
26K
23K
22K
86K
63K
35K
25K
20.5K
19.4K
17.2K
84K
70K
68K
34K
27K
26K
23K
22K

drastically decreased (Figs. 1b, 2b, 3b). From densitometer tracings of the autoradiograms it has been determined that the amount of ^{35}S-methionine incorporated in a 30 min pulse within 90 min after heat shock is always less than 5% of the level in non-heat shocked samples. Some or all of the major heat shock proteins (1) of 84K, 70K, 68K, 34K, 27K, 26K, 23K, and 22K molecular weights (6) are synthesized by 4 hr after the heat treatment and their labeling is maximal around 7 hr or between 7 and 11 hr after heat shock in all cases except one. In the thoracic epithelium from pupae heat shocked at 34 hr, the labeling is maximal between 4 and 7 hr after heat shock (Fig. 1b). At about the same time that the labeling of the heat shock proteins is at or near maximum, the synthesis of most of the normal stage-specific proteins resumes. Synthesis of the heat shock proteins continues until 11 to 16 hr or longer after the heat shock. In some cases, a normally synthesized protein has the same mobility as a heat shock protein, making the distinction of heat-induced synthesis from normal synthesis difficult.*

b) Double heat shock

Figure 1b shows that after a single heat shock to 34 hr pupae, maximum rates of heat shock protein synthesis occur no earlier than 4 hr after the shock, in both thoracic epithelium and brain. In contrast, after a double heat shock of pupae at approximately the same age, high rates of heat shock protein synthesis are achieved within 2 hr after the shock (Fig. 4b).

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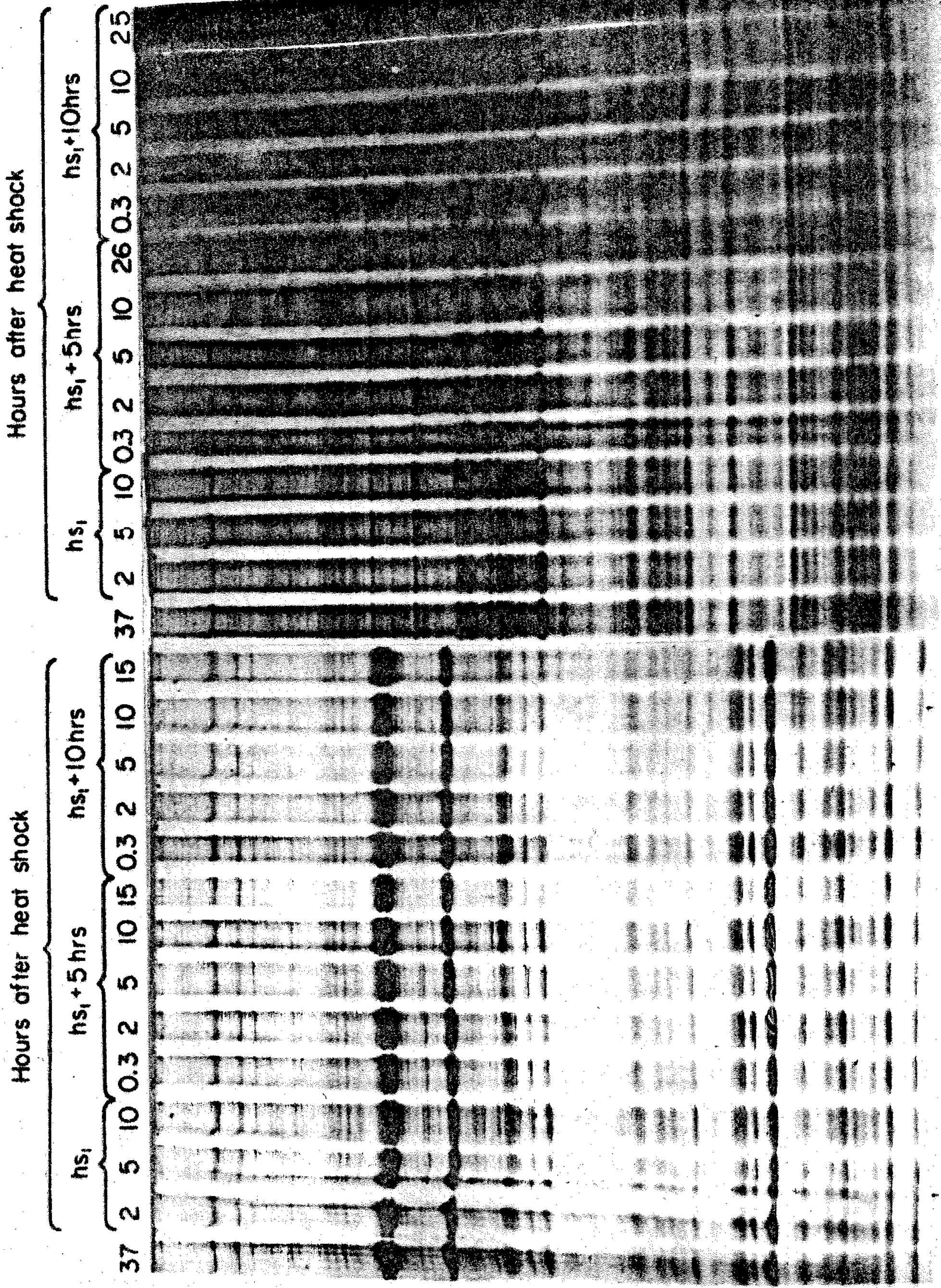
*An exception to the foregoing general description of the time course seems to occur in the thoracic epithelium of pupae heat shocked at 76 hr. This tissue at 7 hr after heat shock has a reduced level of protein synthesis but long-term exposure autoradiograms show the expected proportion of heat shock proteins to one another and to normally synthesized proteins. Hence, this sample may simply have shown poor incorporation of ^{35}S-methionine.

Figure 4. Double heat shock. (a) Coomassie stained pattern and (b) autoradiogram. Electrophoresis was carried out as described in legend to Figure 1. "37" represents the control samples, dissected and labeled 37 hr after puparium formation. The first three lanes after each control sample represent samples taken 2, 5, and 10 hr after a single heat shock of 37 hr pupae; the next group of lanes represent samples taken 0.3, 2, 5, 10, and 15 or 26 hr after a second shock applied 5 hr after the end of the first shock; the next group of lanes represent samples taken 0.3, 2, 5, 10, and 15 or 25 hr after a second shock applied 10 hr after the end of the first shock. The heat shocks in all cases were at 40.2°C for 40 min.

a

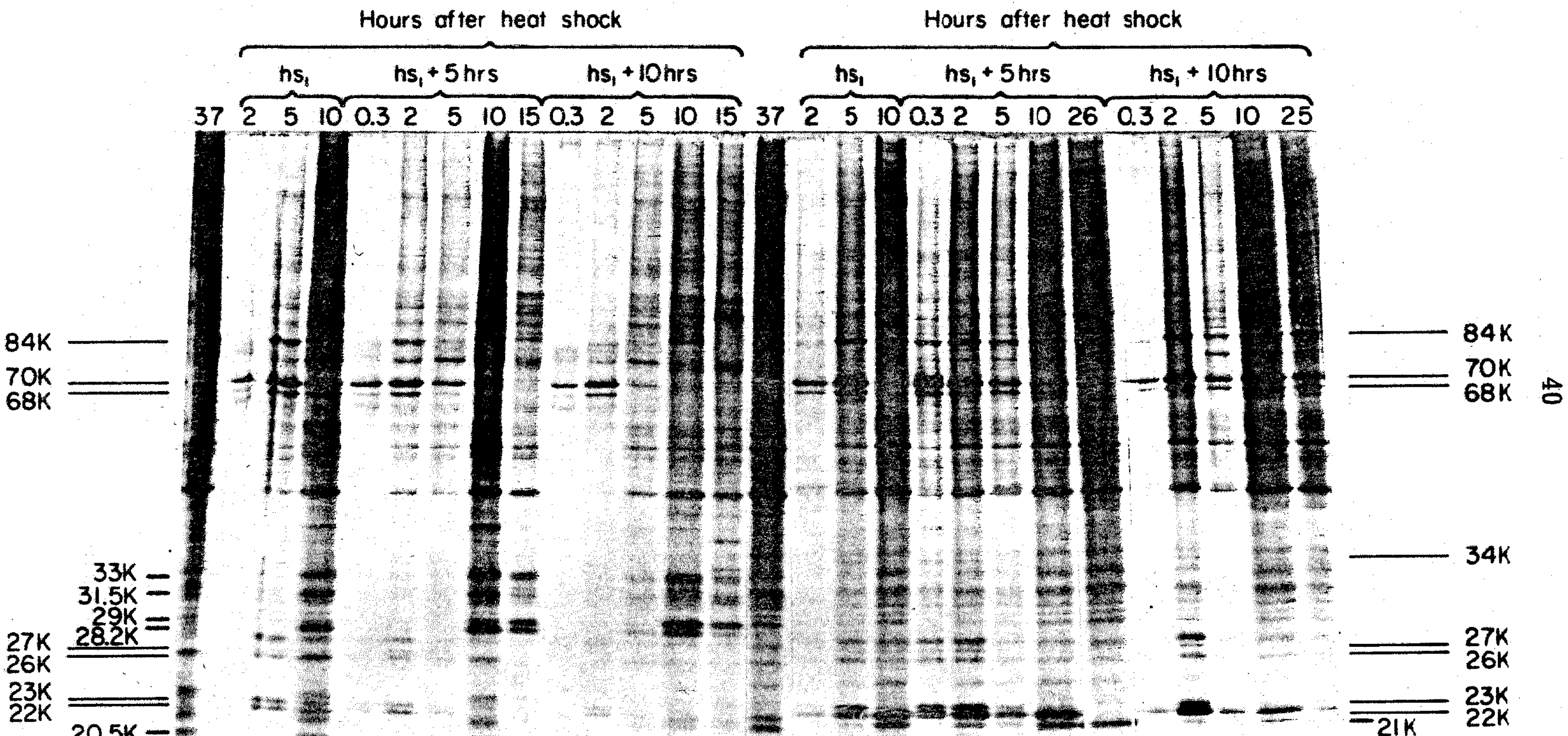
37 hrs

THORACIC EPITHELIUM BRAINS



70K
68K

70K
68K


37 hrs
b
THORACIC EPITHELIUM
BRAINS
Hours after heat shock
Hours after heat shock
hs₁
hs₁ + 5 hrs
hs₁ + 10 hrs
hs₁
hs₁ + 5 hrs
hs₁ + 10 hrs
37 2 5 10 0.3 2 5 10 15 0.3 2 5 10 15
37 2 5 10 0.3 2 5 10 26 0.3 2 5 10 25
84K
70K
68K
33K
31.5K
29K
28.2K
27K
26K
23K
22K
20.5K
84K
70K
68K
34K
27K
26K
23K
22K
21K

More specifically, when the second shock is given 5 hr after the first, a time at which heat shock protein synthesis is proceeding at high rates, the brain continues to synthesize heat shock proteins for at least 2 hr at about the same or at a higher rate. Following the same second shock, the thoracic epithelium, on the other hand, shows an initial decrease in the rate of heat shock protein synthesis before the high rate at 2 hr after the shock.

At 10 hr after a single heat shock, synthesis of normal proteins has resumed at near control rates in the thoracic epithelium and at perhaps somewhat lesser rates in the brain (Fig. 4b). There may still be some ongoing heat shock protein synthesis at this time, in particular synthesis of the 23,000 and 22,000 dalton proteins (see also Fig. 1b). Immediately after a second heat shock given at this time, both the thoracic epithelium and the brain show a sharp reduction in total protein synthesis although synthesis of at least some heat shock proteins is detectable. Protein synthesis reaches maximum rates at a time close to 2 hr after the second shock.

Recovery from heat shock: resumption of synthesis of normal proteins

The thoracic epithelial protein synthesis patterns exhibit many changes during development (14) which allow an estimation of the developmental advancement of pupae after heat shock. The protein synthesis pattern of the brain shows relatively few changes during pupal development (15) and where these occur, they can also be used to estimate the developmental advancement following heat shock. Some of the bands in the autoradiograms which appear and disappear at different ages are marked by molecular weight designations in Figures 1b, 2b, and 3b. The presence or absence of these particular bands is also denoted in Tables 1, 2, and 3.

Table 1. Transiently Synthesized Proteins in Thoracic Epithelium

Pluses and minuses refer to relative intensities of a given band in different lanes of the gel.

	Age Hours after pupariation						Hours after heat shock of 34 hr pupae			
MWt	34	40	48	53	58	62	+11	+16	+25	+29
53K	-	+	+	-	-	-	+	++	-	-
33K	-	-	-	-	+	+	-	-	-	-
31.5K	-	±	+	+	-	-	±	+	+	+
29K	-	-	++	+	-	-	-	+	++	+
28.2K	-	+	++	-	-	-	-	++	-	-
20.5K	±	±	±	+	-	-	±	±	+	+

Table 2. Transiently Synthesized Proteins in Thoracic Epithelium

Pluses and minuses refer to relative intensities of a given band in different lanes of the gel.

	Age Hours after pupariation						Hours after heat shock of 53 hr pupae			
MWt	53	54.5	56	62	68	72	+11	+13	+20	+29
50K	±	±	-	+	-	-	-	-	+	-
38K	++	++	±	+	±	±	-	+	±	±
33K	-	-	-	+	+	-	-	-	+	+
31.5K	+++	+++	++	+	+	+	++	+	±	±
29K	+++	+	-	-	-	-	+++	+	+	+
26K*	-	-	+	+	+	+	-	-	+	+
20K	+++	+++	+	+	+	+	+	+	+	+
17K	-	-	-	-	-	+	-	-	-	±

*This is not a heat shock protein, but a normally synthesized protein which is slightly faster moving than the 26K dalton heat shock protein.

Table 3. Transiently Synthesized Proteins in Thoracic Epithelium

Pluses and minuses refer to relative intensities of a given band in different lanes of the gel.

	Age Hours after pupariation						Hours after heat shock of 76 hr pupae			
MWt	76	79	83	88	93	98	+11	+16	+22	+29
86K	-	-	+	+	+	+	-	-	-	+
35K smear	+	+	+	+	-	-	+	+	+	+
25K	-	-	-	-	+	+	-	-	±	-
20.5K	+	+	+	+	++	++	+	++	++	+
19.4K doublet	+ +	+ +	+ +	+ +	++ ±	++ ±	++ +	++ +	++ +	+ +
17.2K	++	++	++	+	±	±	++	±	±	±

It is possible that a band at a particular position represents one protein at one age and a different protein at the next age analyzed; however, for the purposes of discussion we will treat a band of a particular mobility present in two or more adjacent lanes of an autoradiogram as if it represented the same protein (or proteins).

Some proteins are synthesized for only a short time, so their synthesis after heat shock gives some indication of the developmental stage of the pupae. For example, a 28.2K dalton protein (Fig. 1b) is normally synthesized at a high rate at 40 and 48 hr. In pupae which were heat shocked at 34 hr, this protein appears 16 hr after the shock, at 51 hr after puparium formation, but is not yet detectable at 11 hr after the shock, at 46 hr. Hence, thoracic epithelial development has been arrested for at least 6 hr with respect to this protein. Along the same lines, the appearance of a set of transiently synthesized proteins of molecular weights 33K, 31.5K, 29K, and 28.2K, allows one to correlate the developmental stage of 51 hr heat shocked pupae to non-heat shocked pupae between 40 and 48 hr, and 60 hr heat shocked pupae to 53 hr non-heat shocked pupae (Fig. 1b, Table 1).

Figure 2b and Table 2 illustrate the resumption of the normal program of protein synthesis in thoracic epithelium after a heat shock given to 53 hr pupae. The synthesis of a protein of mobility corresponding to 50K daltons in normal 62 hr pupae but not in 56 hr or 72 hr pupae provides a marker for that particular developmental stage. 68 hr pupae also synthesize this protein, but at a much lower rate. The same protein is labeled 20 hr after heat shock (in 74 hr pupae) at a rate comparable to that in normal 62 hr pupae, and certainly greater than that in 68 hr pupae. This protein is not synthesized in 67 hr or 83 hr heat shocked

pupae. Furthermore, a 33K protein, labeled in 62 hr and 68 hr pupae but not in 72 hr pupae, is labeled also in 74 hr and 83 hr heat shocked pupae. Thus 74 hr heat shocked pupae (20 hr after heat shock) are no more advanced than 68 hr normal pupae with respect to the 50K protein, and 83 hr heat shocked pupae (29 hr after heat shock), not as advanced as 72 hr normal pupae with respect to the 33K protein. In the latter case, a developmental delay of at least 11 hr is indicated.

Some non-coordinate resumption of the synthesis of normal proteins can be observed here. Figure 2b and Table 2 show a 29K protein and a 31.5K protein as being strongly labeled, both to the same degree, at 53 hr, and the latter labeled strongly also at 54.5 hr. After heat shock, the 29K protein appears as a strongly labeled band in the sample taken 11 hr after heat shock, but the 31.5K protein is never seen as intensely labeled as the 29K protein.

A 20K protein, also labeled at a high rate at 53 and 54.5 hr (Fig. 2b) is not observed to label as much in the heat shocked samples. In this case, however, intense labeling after heat shock may have occurred between 7 and 11 hr after heat shock, and may, therefore, not have been observed.

The autoradiograph of the brain samples (Fig. 2b) shows clearly changes in only one molecular weight region during this period. A triplet of proteins of mobility corresponding to approximately 70K daltons is labeled from 53 hr to 80 hr, but the relative intensities of the bands comprising the triplet change during this period. At 53 hr the slowest moving band is the faintest in intensity, while the center band is the strongest. By 80 hr the center band is the weakest and the fastest moving band the strongest. The heat shocked pupal brain samples show, at 29 hr after heat treatment, a pattern which looks similar to 72 hr normal pupal brains and at 40 hr after heat treatment, the pattern exhibited by 80 hr

pupae. Thus brains from 83 hr heat shocked pupae have not reached the developmental stage of brains from normal 80 hr pupae, and may be equivalent to brains from 72 hr pupae.

Figure 3b shows the recovery protein synthesis of pupae heat shocked at 76 hr. This figure and Table 3b show examples of non-coordination in the way in which the program of the synthesis of normal proteins is resumed. A thoracic 25K protein not synthesized normally until 93 hr, is labeled at a low rate at 22 hr after heat shock. A 20.5K protein seems to reach maximum intensity at 93-98 hr, but in heat shocked pupae already shows relative maximum intensity at 16 to 22 hr after heat shock (at 93-99 hr). The intensity of this protein has decreased by 29 hr after heat shock. Consider also a pair of adjacent bands, the upper one of which is labeled 19.4K (Fig. 3b). From 76 to 88 hr the two bands seem to share equal intensity whereas at 93 and 98 hr the upper band shows relatively more intensity. In samples taken from heat shocked pupae the upper band shows higher intensity from 11 hr after heat shock to 22 hr after heat shock (from 88 to 99 hr). At 29 hr after heat shock the two bands again have equal intensity. Thus a protein of 25K daltons normally synthesized by thoracic epithelial cells in 93 hr and 98 hr pupae is synthesized only at a low rate in 99 hr heat shocked pupae and not at all in 93 hr heat shocked pupae while 93 hr heat shocked pupae already show two other changes normally associated with 93 hr normal pupae. At the same time, an 86K dalton protein which first appears in normal pupae at 83 hr is not detectable in heat shocked pupae until 29 hr after heat shock (at 106 hr).

Double heat shock

After a double heat shock, resumption of the synthesis of normal proteins

begins between 5 and 10 hr after the second heat shock (Fig. 4b), but probably no sooner than after a single heat shock.

The transiently synthesized proteins normally seen in the thoracic epithelial tissue of 48 hr pupae (Fig. 1b) are synthesized also after the double heat shocks (Fig. 4b) and give some indication of the degree of developmental progression after heat shock. Ten hours after the single heat shock given to 37 hr pupae, only the 28.2K, 31.5K, and 33K proteins are synthesized. Presumably synthesis of the 29K protein has not yet begun. Ten hours after a double heat shock, the 29K protein is synthesized, indicating that the pupae are more advanced than pupae 10 hr after the single heat shock. Furthermore, from the absence of the 31.5K protein at 10 hr after the 10 hr interval double heat shock, and the relative intensities of the 29K and 28K proteins in this sample, one can deduce that the pupae used for this sample were developmentally more advanced than pupae 10 hr after the 5 hr interval double shock.

Coomassie stained patterns

Figures 1a, 2a, and 3a show that heat treatment affects in addition to the protein synthesis patterns, the disposition of proteins which are in concentrations high enough to be detected by staining of the gel. At 58 hr, a 67K protein in thoracic epithelial cells begins to decrease in concentration and by 72 hr is no longer detectable (Figs. 1a, 2a). Pupae heat shocked at 34 hr have not yet begun to lose this protein by 29 hr after heat shock (age 64 hr) (Fig. 1a) while pupae heat shocked at 53 hr begin to lose this protein at perhaps 13 hr after heat shock (age 67 hr) (Fig. 2a). Almost concomitant with the disappearance of this 67K protein is the accumulation of a protein of 43K molecular weight. Pupae

heat shocked at 34 hr have not accumulated any detectable amount of this protein at 29 hr after heat shock (Fig. 1b). Pupae heat shocked at 53 hr show accumulation of this protein at 20 hr after heat shock (age 74 hr) but not at 13 hr after heat shock (age 67 hr) (Fig. 2b). Two other proteins which increase and decrease in concentration at about the same time as the above mentioned changes are the 30K and 24.6K molecular weight proteins, respectively.

Thoracic samples from 76 hr pupae show a band representing a protein of 53K molecular weight as a more intensely staining band than the protein migrating just behind it (Fig. 3a). In the 93 hr sample, this band is less stained than the higher molecular weight band. The change in the relative intensities of the two bands is not seen in heat shocked pupae until 29 hr after heat shock (at 106 hr), giving some indication of the delay in development.

Pupal brain samples do not show any developmentally associated changes in the staining pattern of proteins, but bands of molecular weight 70K and 68K daltons appear in samples from heat shocked pupae in all cases discussed. Figure 4a shows this phenomenon most clearly and also demonstrates the different relative intensities of the two bands, depending on the heat shock conditions and the time elapsed after the last heat shock.

Phenocopies

The conditions for heat shock used in these experiments are known to produce high yields of phenocopies with good survival rates of the animals (12). Timed animals not used for dissection were allowed to develop to the adult stage and inspected for the occurrence of phenocopies. Table 4 presents the types of phenocopies produced and the yields obtained. Figure 5 shows an example of the

Table 4. Yields and Types of Phenocopies after Heat Shock

Age of pupae at time of heat shock	Phenocopy type	Number of flies showing phenocopy per number of eclosed flies
34 hr	angle bristle	51/160
53 hr	twisted bristle	141/151
76 hr	blond tipped bristle	8/8
76 hr	curly blond bristle	1/39
	light bristle	39/39*
37 hr	angle bristle	28/29
37 hr, 43 hr	angle bristle	5/5
37 hr, 48 hr	angle bristle	23/23
	smooth bristle	23/23

*39 includes the set of 8 blond tipped bristles above; blond tipped bristles were not scored for the set of the remaining 31 flies.

Figure 5. Scanning electron micrograph of scutellar bristle of angle bristle-smooth phenotype. This bristle was taken from a fly which had been exposed to 40.2°C for 40 min at 37 hr and at 48 hr.

combination angle bristle-smooth phenocopy which resulted from two heat shocks given 10 hr apart. Those pupae that did not eclose always displayed the extreme phenocopy phenotype.

Discussion

Heat shock response in pupal thoracic epithelium and pupal brain is similar in the two tissues and at three different ages

With regard to regulatory mechanisms, it is of interest that the brain and thoracic epithelium show a similar time course for the induction of heat shock protein synthesis and the repression of and resumption of normal protein synthesis. After a 40.2°C heat shock for 40 min, both tissues shut down translation immediately after heat shock and for at least 1 hr after the heat shock so that the level of ^{35}S-methionine incorporation into protein in this period is never more than 5% of the level before heat shock. Synthesis of heat shock proteins can be detected at low levels as early as 1 hr after heat shock but these proteins are usually not labeled at a maximum rate until after 7 hr following heat shock, and normal protein synthesis does not resume at the control rate usually until after 7 hr following heat shock (Figs. 1b, 2b, 3b).

The brain is one of the few larval tissues to survive metamorphosis. A few morphological changes of the brain and thoracic ganglion do take place during pupal life, and towards the end of this period extensive innervation of the muscles and sensory structures must take place, perhaps affecting micro structure of the brain and ganglion. Nevertheless, the brain seems to have a fairly constant program of gene expression: the pattern of protein synthesis changes from 34 to 98 hr in only a few components. A more extensive analysis of protein synthesis patterns of developing nervous tissue in Drosophila is in preparation (15).

In contrast to the brain, the thoracic epithelium only begins to grow and differentiate after pupariation. The epithelium is derived from several imaginal discs; it rapidly differentiates to several kinds of cells, among which are bristle cells. The pattern of protein synthesis in thoracic epithelial cells undergoes many changes, with some proteins being synthesized for only a few hours or less (Figs. 1b, 2b, 3b; 14).

That both tissues cease production of the normal proteins for approximately the same length of time and label heat shock proteins at approximately the same time again points to some common mechanism for the induction of heat shock gene expression which supersedes normal gene function (1).

Heat shock conditions which produce phenocopies have a stronger effect on protein synthesis

The heat shock effect on protein synthesis has been reported so far for embryonic, larval, prepupal and adult tissues of D. melanogaster (1, 2, 20). No pupal tissues have yet been analyzed after heat shock; furthermore most heat treatments used for analysis of protein synthesis patterns after heat shock were 20 min at 37° or 37.5°C. Under these conditions, prepupal and larval tissues synthesize the heat shock proteins at a high rate immediately after the heat shock and, if kept at 25°C, after the heat shock, decrease their synthesis to undetectable levels by 3 to 4 hr after heat shock (2, 21).

We have shown here the response to a 40.2°C, 40 min heat shock in pupal tissues to be heightened and prolonged relative to the effect in larval tissues heat shocked at 37°C for 20 min (2). The heat shock proteins in pupal brain and thoracic epithelium are not synthesized at a maximum rate, in general, until after 7 hr

following heat shock. This difference in the time course of the response is largely due to the difference in the severity of the heat treatments, rather than to the difference in the developmental age of the animal. In fact, brain and thoracic epithelium from pupae heat shocked at 37.5°C for 20 min synthesize the heat shock proteins immediately after heat shock and at a very low rate and not at all, respectively, by 7 hr after the heat shock (data not shown).

The more severe heat shock at the same time drastically reduces total protein synthesis to $\leq$ 5% of that before heat shock, for at least 1 hr after the heat shock. In contrast, the milder heat shock reduces total protein synthesis by no more than 50% in brains and 80% in thoracic epithelial tissue of 34 hr pupae. In these cases, the heat shock bands in the autoradiograms are superimposed on backgrounds of normally occurring bands.

The difference in the effects of different temperatures has relevance to a discussion of phenocopies. Phenocopies are produced in pupae at several ages at a high penetrance by a 40.2°C shock for 40 min (12) whereas 34 hr pupae heat shocked at 37.5°C for 20 min showed no phenocopies (13). Prepupae, in contrast to pupae, do not make good subjects for heat-induced phenocopies. They do not survive a heat treatment of 40°C for 40 min and after less severe treatment, e.g., 40°C for 20 min, yield phenocopies at low penetrance (13).

It is possible, then, that the severe repression of normal protein synthesis caused by more severe heat shock conditions plays an important role in the production of phenocopies. After a 40.2°C, 40 min shock, protein synthesis is so much reduced that it could either result in the uncoordinated synthesis of some constantly synthesized and transiently synthesized normal proteins or enhance such effects that would occur to a lesser extent with a milder heat shock. We have

shown the most clearcut examples of non-coordinated synthesis of normal proteins in thoracic epithelium. The number of labeled polypeptides, about 70, we can detect in one lane of the polyacrylamide gels we have used may be less than 10% of the number of labeled species actually present. O'Farrell (22) has claimed that 1000 E. coli proteins can be detected by autoradiography of a two-dimensional isoelectricfocusing-SDS electrophoresis gel. Thus other examples of non-coordinated synthesis of normal proteins or even the omission of one or more species might not be detected on these one-dimensional gels. The time intervals between samples were necessarily large, usually $\geq$3 hr; consequently, some cases of mistimed expression of particular genes could have been missed if the timing were off by less than the intervals between samples.

It has been proposed, as an explanation for phenocopy production, that after heat shock, a gene normally only transiently expressed at about the age at which the heat shock is given, fails to be active or is not active at the precise time at which the gene product is required for normal development (10). Another explanation is that the transiently expressed gene functions at the right time but in an incomplete or incorrect environment as a result of the heat shock. In fact, Ashburner (9) has observed that after a prolonged heat shock to larvae, 37.5°C for 4 hr, salivary gland chromosome puffing activity was nonexistent, and when it did return, after several additional hours, the puffs occurred in abnormal combinations.

The substantial decrease in protein synthesis for at least 1 hr after a 40°C, 40 min heat shock could possibly be due to damage to the cells from the high temperature. Mitchell and Lipps (12) have shown that the level of transcription as well is decreased after heat shock. Normal respiratory activity is affected

after heat shock (for reviews see 23, 24), and the processes of normal gene expression could be postponed until metabolic functions returned to normal. Alternatively, enzymes or proteins in the transcriptional or translational apparatus could be heat sensitive and thereby delay macromolecular synthesis.

Heat shock of 40.2°C for 40 min in 34 hr pupae causes delay in development of about 5 hr

The approximately 5 hr to 10 hr delay in the sequence of normal changes in the thoracic tissue might be expected to coincide with, or represent, a similar delay in development, and in fact, this is substantiated for the lower figure by two facts reported here and elsewhere. Mitchell and Lipps (12) reported that pupae heat shocked at 34 to 37 hr at 40.0°C for 40 min, were delayed in eclosion by 4.3 hr. Also, the same authors have shown, as we have here, that in giving a second heat shock 5 hr after the first, only one phenocopy type is produced. Particular phenocopy types are unique to narrowly delimited periods of development, and, had the animals developed 5 hr since the first heat shock, two phenocopy types would have been produced. In fact, pupae heat shocked twice, 10 hr apart, at 40.2°C for 40 min, developed into individuals exhibiting two phenocopy types (Table 4). Thus, development, as measured by sensitive periods for phenocopy types, has proceeded somewhat by 10 hr after a heat shock. Lindsley and Poodry (25) have also reported developmental delays caused by heat shock in pupae.

Heat shock protein synthesis is not temporally related to resumption of normal protein synthesis

When pupae are heat shocked a second time, 5 or 10 hr after the end

of the first heat shock, a high rate of labeling of the heat shock proteins occurs at 2 hr after heat shock (Fig. 4b). Yet, the rate of the resumption of normal protein synthesis is comparable to the rate after a single heat shock (Fig. 4b). Thus it appears that heat shock protein synthesis and return to normal protein synthesis are under separate control.

Heat shock protein synthesis may be reinduced faster after a 10 hr interval second shock because the messengers may still be present, and requirements for their translation may still be met. Nevertheless there is again a decrease in total protein synthesis immediately after 10 hr interval double heat shock, which may be due to some repeated impairment of metabolic function or of translation. In contrast, just after a second heat shock is given 5 hr after the first, the brains continue to synthesize heat shock proteins at a high rate. Apparently in this case, heat shock translation in progress is resistant to the usual damping influence a heat shock has on protein synthesis.

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CHAPTER 2

SITE OF SYNTHESIS OF THE HEAT SHOCK PROTEINS

Introduction

Since heat shock puffs can also be induced by uncouplers of oxidative phosphorylation (1), inhibitors of electron transport (1-3), and during recovery from anoxia (2), mitochondrial electron transport and oxidative phosphorylation have been suggested as possible primary targets of inducers of the heat shock response (4). Heat treatment was observed by Johnson (5) to alter mitochondrial morphology in *Calliphora*. Sherman (6) induced petite mutants in yeast by heat treatment. That elevated temperatures should affect mitochondrial structure and function is not surprising if one considers the possible effect on membrane fluidity and consequently on permeability and lipoprotein structures. The synthesis and assembly of mitochondrial enzyme complexes could be drastically affected, as well as electron transport and oxidative phosphorylation.

One question that could easily be answered is whether any of the heat shock proteins are mitochondrially synthesized mitochondrial proteins. Mitochondria in animal cells synthesize three of the seven subunits of cytochrome oxidase (7, 8) and four of the nine subunits of the oligomycin sensitive ATPase (7). By labeling in the presence of specific inhibitors of cytoplasmic or mitochondrial translation, the site of synthesis of the major heat shock proteins could be determined.

Materials and Methods

Fly culture

The Oregon R stock of wild-type *Drosophila melanogaster* was used for

these experiments. Mass culture and synchronization by flotation were carried out as described by Mitchell and Mitchell (9).

Drug treatment

Following heat treatment (37.5°C for 20 min) of prepupae, salivary glands were dissected in "A" medium (10) and then incubated in "A" medium containing 100 μg/ml emetine, or 100 μg/ml chloramphenicol, or 1 μg/ml ethidium bromide for circa 7 min or circa 25 min, depending on the experiment.

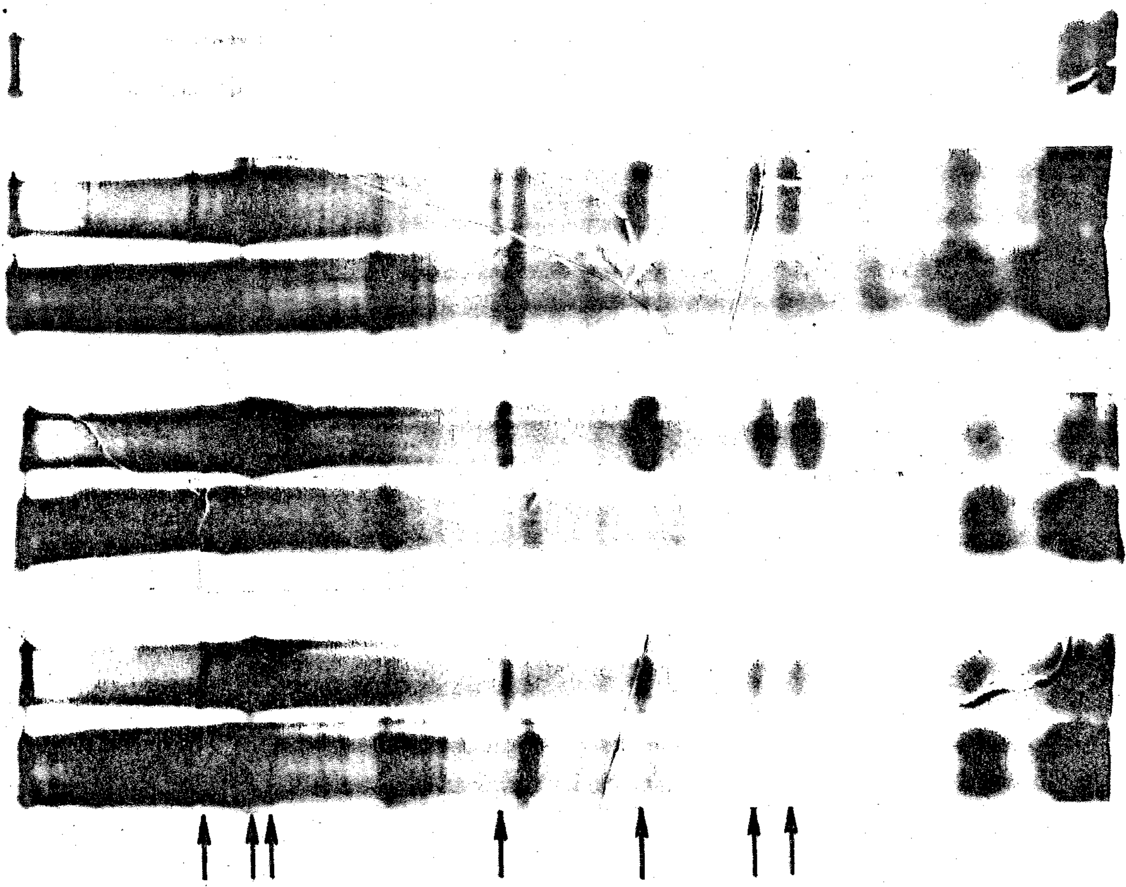
Following the preincubation, the glands were immediately transferred to about 4 μl "A" medium containing 2-6 μCi ^{35}S-methionine (~300 Ci/mMole, New England Nuclear) (the higher concentrations were used with emetine) and the drug. After labeling for 20 to 29 min, the incorporation was terminated by the addition of 10% TCA and the glands were prepared for gel electrophoresis as described in Chapter 1a. Control samples were labeled for 20 min, immediately after dissection, in "A" medium containing ^{35}S-methionine.

Portions of each sample were counted in a scintillation counter and dilutions were made so that between 10,000 and 50,000 cpm were run in each lane of the gel.

Results and Discussion

Figure 1 shows that heat shock protein synthesis is completely inhibited by emetine, a specific inhibitor of cytoplasmic protein synthesis (11, 12) and not at all by chloramphenicol or ethidium bromide, both of which are specific inhibitors of mitochondrial protein synthesis (12, 13).

Figure 1. Protein synthesis in the absence or presence of drugs. Salivary glands were labeled in the absence of drugs (Control), in the presence of 100 μg/ml chloramphenicol (CAP), in the presence of 1 μg/ml ethidium bromide (EthBr), or in the presence of 100 μg/ml emetine (Emetine). The left lane in each pair represents glands from prepupae kept at 25°C, and the right land, glanes from prepupae heat shocked at 37.5°C for 20 min. Glands were preincubated with inhibitor for 7 min before labeling in the presence of the same inhibitor.


Emetine
Eth Br
C A P
Control

Since the permeability to chloramphenicol and to ethidium bromide in salivary gland plasma and mitochondrial membranes has not been established, failure to inhibit heat shock protein synthesis by these drugs would not stand alone as compelling evidence that the heat shock proteins are synthesized on cytoplasmic ribosomes. However, the complete inhibition of the synthesis of the heat shock proteins by emetine does establish the cytoplasmic ribosomes as their site of synthesis.

One could also ask whether inhibition of mitochondrial protein synthesis could also induce heat shock gene activity. Barath and Küntzel (14, 15) have observed in yeast that inhibition of the synthesis of mitochondrially synthesized proteins stimulates the production of some cytoplasmically synthesized mitochondrial proteins. Labeling of salivary glands continues for at least 90 min after dissection and incubation in medium "A" but with a gradually decreasing rate (16). With this restriction in mind non-heat shocked salivary glands were preincubated in the presence of 100 μg/ml chloramphenicol or 1 μg/ml ethidium bromide for 22 or 29 min, respectively, and then labeled in the presence of the same drug for 29 or 20 min, respectively.

No heat shock protein synthesis was induced by this treatment; however, this result does not rule out the possibility that depletion of mitochondrially synthesized subunits of mitochondrial enzymes might eventually induce heat shock puffing and heat shock protein synthesis. If the turnover of mitochondrially synthesized proteins is low, or a large pool of these proteins exists, then long-term exposure to inhibitors would be necessary to see any possible effects. This long-term exposure could be effected by injecting the drug into larvae and observing

at subsequent intervals of time the proteins labeled in excised tissues. Drosophila tissue culture cells could also be used for these inhibition studies.

Very little emetine-resistant protein synthesis could be detected in glands from either control or heat shocked prepupae. The washing of the glands included washing with a 1:1 mixture of chloroform and methanol (16). Such a solvent has been reported to extract mitochondrial proteins to a variable extent (17). Hence from this experiment one cannot say anything about the behavior of mitochondrial protein synthesis after heat shock. It may be stated here that none of the major heat shock proteins is extracted by the chloroform-methanol mixture (H. K. Mitchell, personal communication.

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CHAPTER 3

PHENOL OXIDASE ACTIVITY AFTER HEAT SHOCK

Introduction

Information regarding the physiological role of any of the heat shock proteins could provide some clues as to the mechanism of the induction of puffing or, at least, the identification of the effector molecule(s).

Koninkx (1) has suggested an NADH dehydrogenase activity for at least one of the heat shock proteins, since the enzyme activity increases after heat shock, and the active components comigrate, on nondenaturing gels, with components whose labeling with ^{35}S-methionine is increased after heat shock. Sin and Leenders (2) have found an increase in the mitochondrial isocitrate dehydrogenase activity after heat shock. In neither case have the subunit sizes of the enzyme been determined and the subunits correlated with any particular heat shock proteins.

Johnson (3) observed a darkening of Calliphora pupae beginning one day after a 3 min heat shock of unspecified temperature. He suggested that the explanation was an imbalance between phenol oxidases and (hypothetical) dehydrogenases which acted as inhibitors of the phenol oxidases. An independent observation was made in this laboratory by H. K. Mitchell, that phenol oxidase activity was increased in homogenates of pupae heat shocked at 40°C for 40 min.

Phenol oxidase is a developmentally regulated enzyme which plays an important role in sclerotization and melanization. The enzyme in vivo is held in an inactive form and is activated at localized sites in the animals. It inactivates

itself, presumably as a consequence of its activity in cross-linking tyrosine residues (for review, see 4).

Seybold et al. (5) have determined the minimum number of components necessary to produce an active enzyme. Two components, P' and A (see below), constitute the active enzyme. P' is derived from its inactive precursor, P, by the action of S, which in turn is derived from its inactive precursor, pre-S, by the action of S-activator.

The A components are heterogeneous. Three components, A_1, A_2, and A_3, have been separated on nondenaturing gels and have been shown to confer at least two different specificities on the active enzyme (6). The enzyme in vitro forms aggregates as it activates, thereby allowing partial purification by equilibrium density gradient centrifugation (7).

The possibility of a phenol oxidase activity for one of the most strongly labeled heat shock proteins, i.e., the 70,000 or 68,000 dalton protein, was investigated by determining whether the radioactivity from heat shocked cells would copurify with phenol oxidase in a sucrose gradient.

Materials and Methods

Fly stocks

Wild-type and ebony stocks of Drosophila melanogaster were used as sources of enzyme activity. The wild-type stock was used to prepare labeled salivary gland proteins.

Heat shock

Heat shocks were given at 37.5°C or 40°C, for 20 min or 40 min, respectively.

Preparation and assay of phenol oxidase

For the data presented in Figures 2 and 3, total phenol oxidase activity in a homogenate of 36-37 hr pupae was measured as described by Mitchell (8) except that 100 mg of pupae were ground in 2.25 ml buffer and the concentration of dopa (3,4-dihydroxyphenylalanine) used in the assay was 1 mg/ml. Maximum enzyme activity was reached usually within 90 min.

The preparation of the enzyme and centrifugation of the enzyme through sucrose gradients were carried out as described in detail by Mitchell et al. (7) except that sample quantities were usually 1.7 g. The procedure used in these experiments is essentially as follows: the pupae were ground with half the sample weight of sand in 2 ml buffer. The slurry was diluted and then centrifuged to remove insoluble materials. The supernatant was brought to 56% saturation with ammonium sulfate. The precipitate contains all of the original potential enzyme activity of the crude extract but only 10% of the soluble protein (7). The pellet was washed with 56% saturated ammonium sulfate into two centrifuge tubes and repelleted. At this point 16-18 salivary glands which had been dissected from heat shocked or control larvae or prepupae and which had been pulse-labeled with 0.5 mCi of ^{35}S-methionine for approximately 30 min were ground in 0.1 ml MOPS buffered medium (9). To this homogenate was added 0.75 ml 1.5 M KCl. Each of the two homogenates was used to resuspend one of the ammonium sulfate precipitated pellets. At this salt concentration the enzyme is inactive. Insoluble material was pelleted at 35K rpm for 30 min in a Beckman 40 rotor and the supernatant passed over a 5 ml bed volume Sephadex G-25 column equilibrated with 0.02 M phosphate buffer at pH 6.7. The KCl and the unincorporated methionine

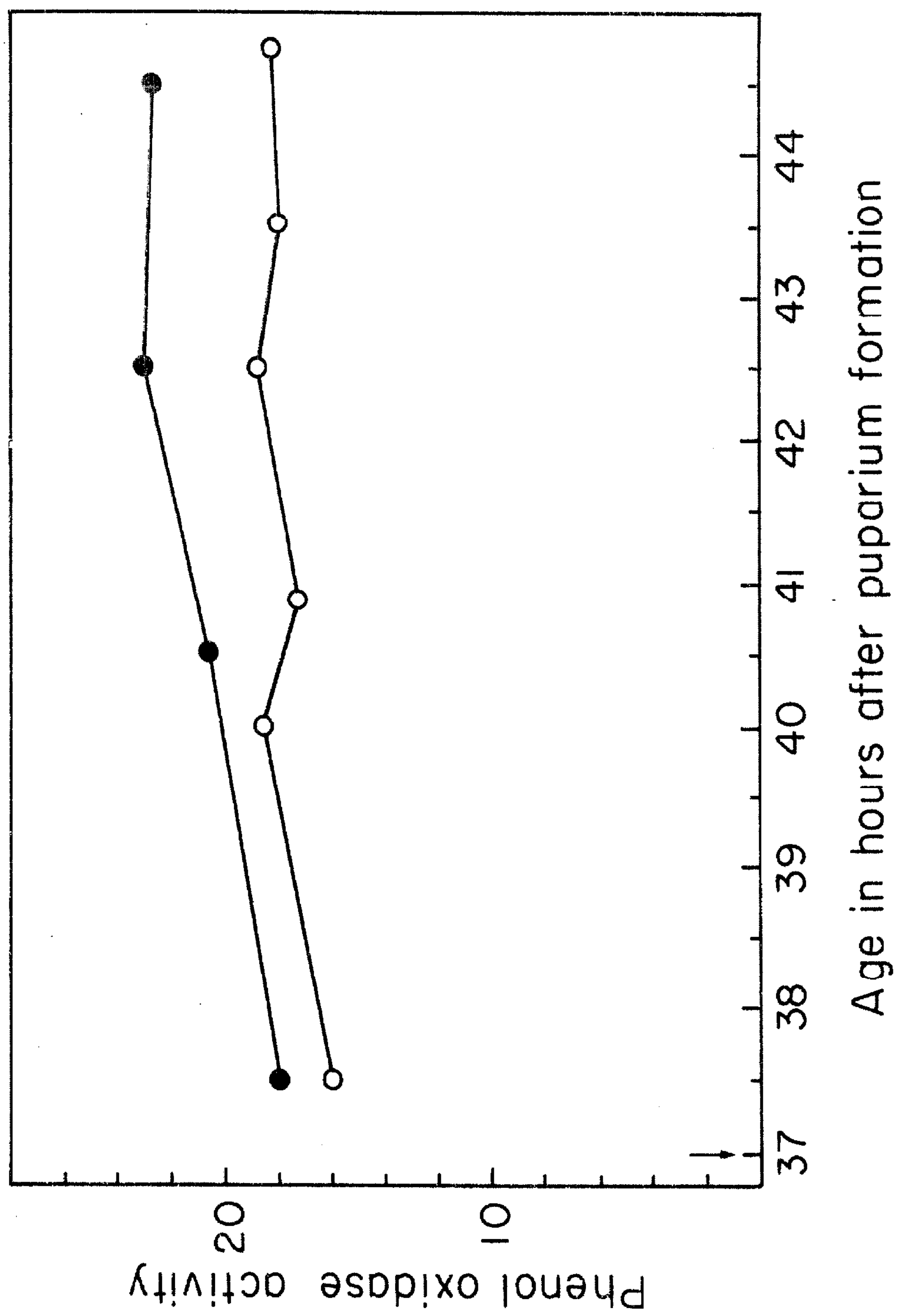
are separated from the protein which comes through in the void volume. The enzyme activation was monitored and after 2 to 2.5 hr, run on a 30 ml sucrose gradient formed with heavy and light solutions prepared by dissolving 1.8 g and 0.7 g sucrose, respectively, per ml 0.02 M phosphate buffer (pH 6.7). The centrifugation was carried out at 12,000 rpm for up to 17 hr in an SW25.1 Beckman rotor. Fractions of 0.5 to 0.6 ml were collected from the bottom of the tube in the cold. Usually 100 µl portions of fractions were counted in a mixture of 1 ml H_2O and 10 ml Aquasol (New England Nuclear). Fifty µl portions of fractions were assayed for phenol oxidase activity using a 1 mg/ml dopa solution.

Results and Discussion

Figure 1 shows the increase in phenol oxidase activity after a 40°C, 40 min heat shock. The increase builds up slowly after heat shock and by 5.5 hr, the heat shocked samples show 25% more activity than the control samples.

If this increase in activity is the result of de novo synthesis, specifically synthesis of heat shock proteins, then pulse-labeled proteins from heat shocked cells should copurify with the enzyme to a greater extent than pulse-labeled proteins from control cells. Since some heat shock proteins only incorporate about 2% of the label, the possibility of detecting a phenol oxidase activity for a protein is more likely if the protein incorporates $\geq$10% of the label. Such candidates are the 70,000 and perhaps the 68,000 dalton proteins which together incorporate 26% of the label in a 20 min pulse immediately after a 20 min heat shock at 37.5°C (Chapter 1a). Activated phenol oxidase from mixed larvae and prepupae separates into two or three bands on an equilibrium sucrose density gradient (7). The lower

Figure 1. Phenol oxidase activity after a heat shock of 40°C for 40 min given to 36 hr pupae. The activity is denoted as 100 times the maximum change in $OD_{475\ nm}$ per minute in 0.6 ml of a 1 mg/ml dopa solution caused by the addition of 50 μl crude extract containing the enzyme (see also Materials and Methods). The arrow marks the end of the heat shock. Open circles, non-heat shocked controls; closed circles, heat shocked pupae.

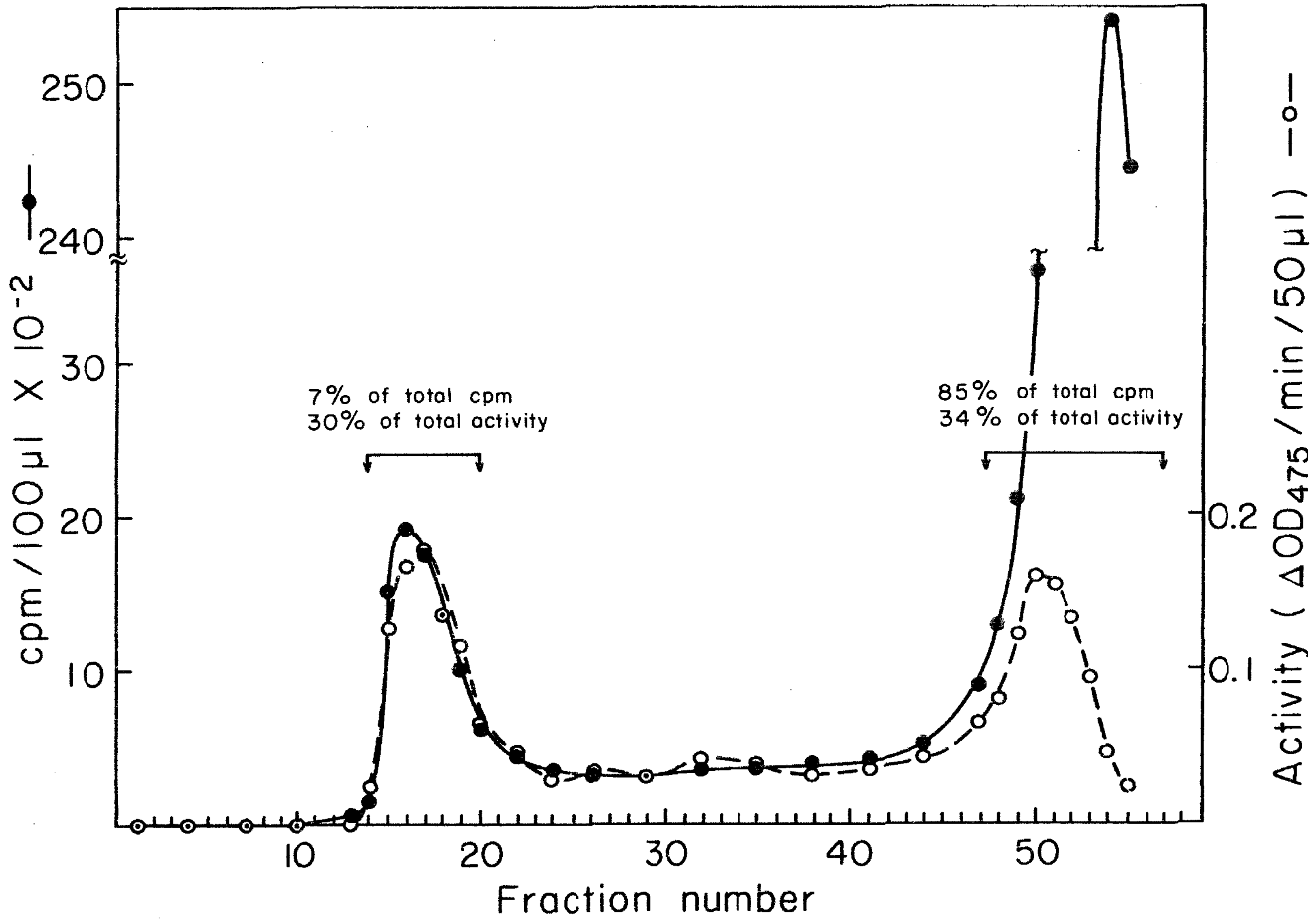

Phenol oxidase activity
20
10
37
38
39
40
41
42
43
44
Age in hours after puparium formation

band migrates to a position one-third up from the bottom of the tube; the third band, seen occasionally, migrates to somewhere above this position, and the uppermost band migrates to just below the top of the gradient. Soluble proteins remain at the top of the gradient.

Figures 2 and 3 show the equilibrium density gradient profiles of two mixtures of labeled proteins and phenol oxidase. For the gradient represented in Figure 2, the pulse-labeled proteins from non-heat shocked prepupal salivary glands were mixed with half of a preparation of phenol oxidase from mixed larvae and prepupae. The enzyme to this point had been kept in an inactive (and unaggregated) state by the presence of high salt (7). The enzyme was then allowed to activate and was centrifuged through a sucrose gradient as described previously (7). The gradient in Figure 3 was formed in the same way except that labeled proteins from salivary glands which had been heat shocked at 37.5°C for 20 min were mixed with the other half of the phenol oxidase preparation.

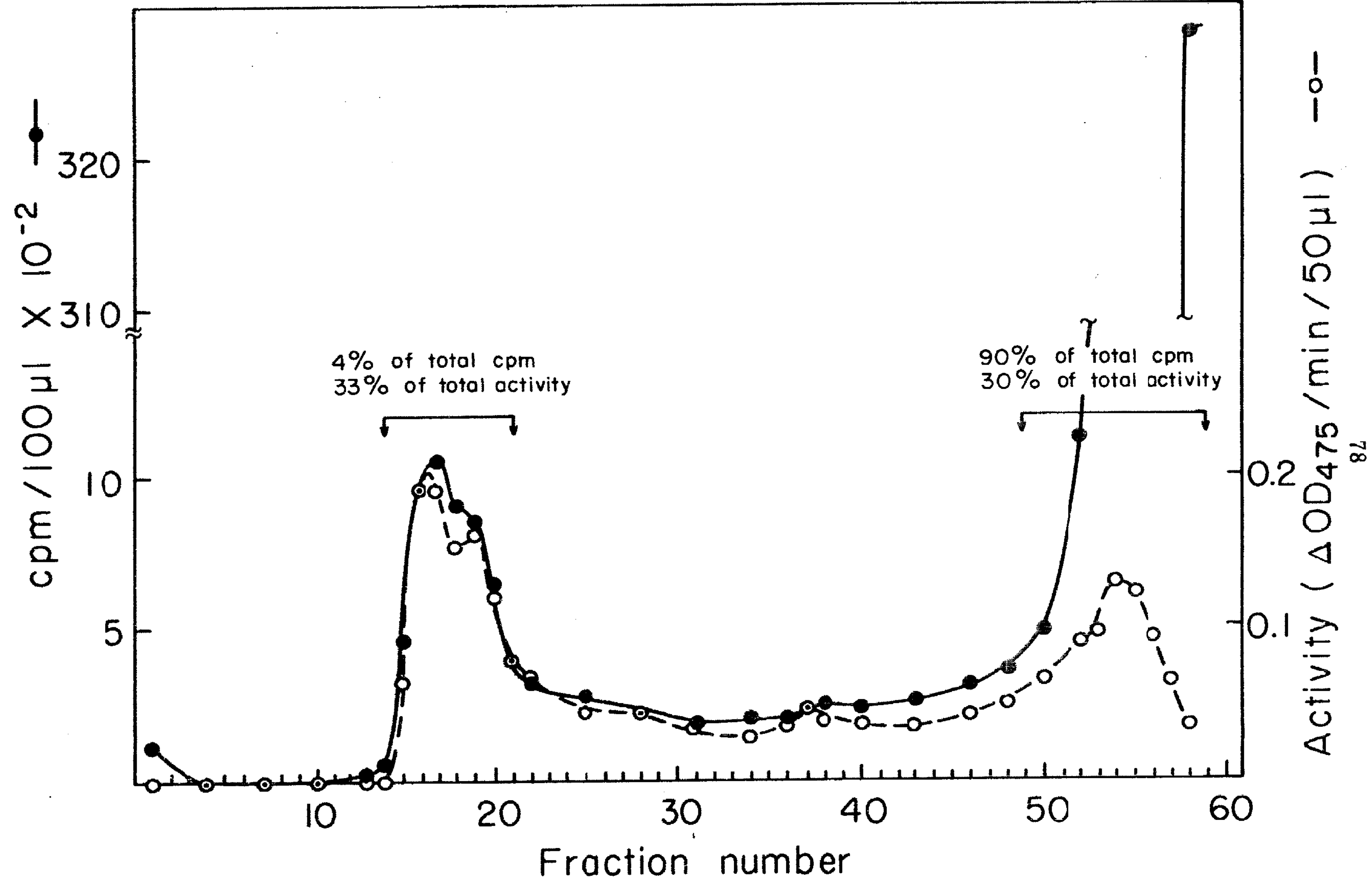
As is denoted on Figure 2, 30% of the enzyme activity is found in the lower band but only 7% of the radioactivity. When labeled proteins from heat shocked cells were mixed with the enzyme, only 4% of the radioactivity, along with 33% of the phenol oxidase activity was found in the lower band. The upper band of phenol oxidase activity is not resolved from the radioactivity at the top of the gradient, so the question of whether the radioactive proteins copurify with the enzyme rests with an analysis of the lower band material. Table 1 lists the results from this and similar experiments. Although the percentage of radioactivity in the lower band varies, the percentage from heat shocked proteins is always lower than from control proteins in a given experiment.

Figure 2. Profiles of radioactivity and phenol oxidase activity in an equilibrium sucrose density gradient. Half of a preparation of phenol oxidase was mixed with labeled salivary gland proteins from larvae kept at 25°C. The enzyme was allowed to activate and was then centrifuged to equilibrium through a 40 to 65% sucrose gradient. Activity is denoted as the change in $OD_{475\ nm}$ per minute after the addition of 50 μl of the fraction to 0.6 ml of 1 mg/ml dopa solution.



cpm / 100 µl X 10-2
250
240
30
20
10
Activity (ΔOD475 / min / 50 µl)
0.2
0.1
Fraction number
10
20
30
40
50
7% of total cpm
30% of total activity
85% of total cpm
34% of total activity

Figure 3. Profiles of radioactivity and phenol oxidase activity in an equilibrium sucrose density gradient. The other half of the preparation of phenol oxidase mentioned in the legend for Figure 2 was mixed with labeled salivary gland proteins from larvae exposed to 37.5°C for 20 min. Centrifugation was carried out as described in the legend to Figure 2 and in Materials and Methods. Activity is denoted as the change in $OD_{475\ nm}$ per minute after the addition of 50 µl of the fraction to 0.6 ml of a 1 mg/ml dopa solution.



cpm / 100 µl X 10^{-2}
320
310
10
5
Activity (ΔOD_{475} / min / 50 µl)
0.2
0.1
4% of total cpm
33% of total activity
90% of total cpm
30% of total activity
10
20
30
40
50
60
Fraction number

Table 1. Comigration of Radioactivity with Phenol Oxidase through Sucrose Gradient

Experiment	Source of phenol oxidase activity	Source of labeled proteins	Heat shocked*	Percent of total phenol oxidase activity in the gradient which is found in the lower band	Percent of total radioactivity in the gradient which is found in the lower band
1	ebony, mixed larvae and prepupae	wild type late larvae	-	40	6
			+	33	3
2**	wild type, mixed larvae and prepupae	wild type late larvae	-	30	7
			+	33	4
3	wild type, mixed larvae and prepupae	wild type prepupae	-	25, 14***	11, 6***
			+	23, 17***	6, 3***
4	wild type, mixed, more larvae than prepupae	wild type 110 hr larvae	-	22	5.7
			+	14	2.5

*Heat shock was 40 min at 40°C.

**Experiment 2 is the same as that described in Figures 2 and 3.

***The second figure represents the value for a third band, of slightly less density in the gradient than the lower band.

These results suggest that labeled proteins from heat shocked cells have less phenol oxidase activity than labeled proteins from control cells. Blumenthal (10) found that, under similar activation conditions, 5% of the radioactivity from ^{14}C-labeled E. coli proteins migrated with the heavy phenol oxidase band in a sucrose gradient. 85% of the radioactivity could be washed with distilled water from the particulate enzyme in this band. To this extent, radioactivity in the lower band material may be the result of nonspecific association with the enzyme.

It is not feasible to purify further the phenol oxidase from the lower band to follow the copurification of counts with the activity since the enzyme cross-links protein in that band and, as a consequence, the enzymatic activity of the material decreases with time (8, 11). Furthermore, Blumenthal (10) succeeded in solubilizing only 75% of freshly prepared and washed lower band material in 98% formic acid and 59% of the lower band material in 9 M urea, 5% SDS, and 5% β-mercaptoethanol.

If, in the experiments described in Figures 2 and 3 and Table 1, nonspecific sticking of the labeled proteins to the phenol oxidase accounts for the comigration of radioactivity with the enzyme activity, then the differences between the heat shock and control samples could be due simply to different degrees of "stickiness" of particular labeled proteins.

Another explanation which does not rule out a phenol oxidase function for a heat shock protein is that the control and the heat shocked cells synthesize different phenol oxidase components. There are at least three different A components (6) each of which is capable of forming an active enzyme with the activated P component. Since the active enzyme is not dissociable, a test of this hypothesis would probably necessitate purification of the A components from pulse-labeled

heat shocked and control cells. If a heat shock component is identical to an A component, radioactivity should coincide with phenol oxidase activity and electrophoresis on SDS gels of the A component should show a protein having a molecular weight corresponding to a heat shock protein. The A component has been purified (5) and when run in a continuous phosphate buffer system on SDS gels (12) has a molecular weight of 77,000 daltons. Since the heat shock proteins of D. melanogaster have not been run on SDS gels in this buffer system it is difficult to rule out a noncorrespondence of heat shock protein to component A (13).

If one rules out nonspecific association of the heat shock pulse labeled proteins to the enzyme, then, since the heavy band contains only ~1/3 of all the phenol oxidase activity, the total percentage of label in phenol oxidase could be as high as 12% or more (3 x 4%). However, the ratio of radioactivity to phenol oxidase activity is not necessarily constant in the different kinds of phenol oxidase separated by sucrose gradient (see Table 1, experiment 3). It has been proposed (7) that the different densities of the particulate enzyme may be due to different ratios of the components, one to another. Hence 12% is only a rough estimate of the possible amount of label represented by phenol oxidase.

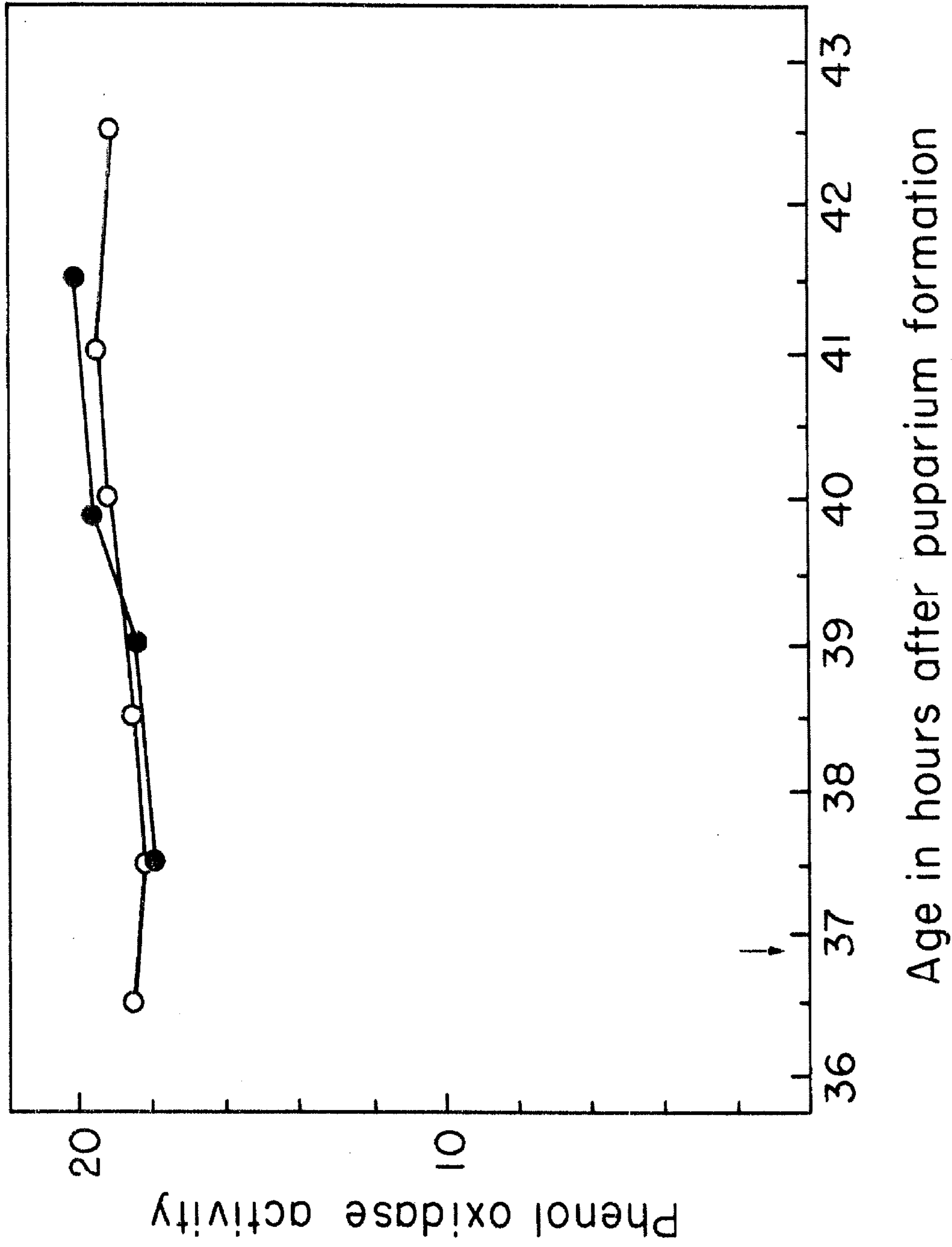
On the basis of available evidence, total phenol oxidase activity depends only on the total amount of A and P components (in the potentially active state) in the animal. It was hypothesized that increased activity must arise from increased synthesis. However, it is also possible that the increased activity is due to a decreased or delayed turnover of the enzyme components in the presence of more or less constant de novo synthesis of the components. Turnover could be the sum of degradation and self-inactivation.

The experiments described in Chapter 1b showed that a shutdown of protein synthesis occurs immediately after exposure of pupae to 40.2°C for 40 min. After about 1 hr, the animals begin to synthesize heat shock proteins at gradually increasing rates. In thoracic epithelial tissue from pupae shocked at 34 hr, the maximum rates occur between 4 and 7 hr after the shock. At about the same time, synthesis of normal proteins resumes. However the synthesis of some normal proteins is not coordinated properly with the synthesis of other proteins; also, the program of stage-specific synthesis of proteins is delayed. Moreover, this heat treatment causes a delay in the stage-specific loss or disposition of some proteins. One could imagine, then, a transient imbalance in the processes of synthesis and turnover, so that enzyme activities such as phenol oxidase would increase after heat shock, but not as the result of markedly increased synthesis.

After a 37.5°C heat shock for 20 min, a shutdown of total protein synthesis is not observed. The heat shock proteins are synthesized immediately after the shock together with normal proteins which, however, are synthesized at a lower rate than normal.

To see whether the severity of the heat shock would in fact affect the increase in phenol oxidase activity, the experiment described in Figure 1 was repeated using a heat shock of 37.5°C for 20 min. The results are shown in Figure 4. No increase in phenol oxidase activity over control activity was found in the homogenates of heat shocked cells. Thus the evidence presented here seems to rule out a phenol oxidase activity for any major heat shock protein. This evidence is not conclusive, however, since turnover rates of the heat shock proteins and of the phenol oxidase components after different temperature treatments have not been determined.

Figure 4. Phenol oxidase activity after a heat shock of 37.5°C for 20 min given to 36 hr pupae. The activity is denoted as 100 times the maximum change in $OD_{475\ nm}$ per minute in 0.6 ml of a 1 mg/ml dopa solution caused by the addition of 50 µl of crude extract containing the enzyme.


Phenol oxidase activity
20
10
36
37
38
39
40
41
42
43
Age in hours after puparium formation

The results presented here suggest another explanation, not cited or mentioned in Chapter 1b, for the way in which phenocopies can be produced. The concentration of phenol oxidase in the animal is affected by a heat shock of 40°C for 40 min, but not by a heat shock of 37.5°C for 20 min. At the same time, the more severe heat shock causes the production of phenocopies, whereas the milder heat shock does not (14). It may be proposed, then, that altered concentrations of enzymes resulting from temporary imbalances in the processes of synthesis and turnover might be responsible for alterations in structures or pigmentation. The production of stage-specific phenocopies would, according to this explanation, result from the requirement, by affected cells, for a particular concentration of an enzyme at a particular stage in development.

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CHAPTER 4

STUDIES ON THE 84,000 DALTON HEAT SHOCK PROTEIN

Introduction

The heat shock proteins are defined by their labeling after exposure of tissues to high temperature, and are distinguished from one another by their molecular weights. Detection of their presence depends mostly on autoradiography. In at least one case (described below), the individual proteins cannot be distinguished one from the other. Heat shock proteins can occasionally be detected by other means; for example, all of the heat shock proteins accumulate in tissue culture cells kept at 37°C to levels detectable by staining (1).

Mitchell and Lipps (2) used autoradiography of salivary gland chromosomes from cells labeled with ^{3}H-isoleucine to determine that the ^{3}H-label after heat shock is distributed all over the chromosomes. However, they did observe localized concentrations of label at some loci. They also showed that extraction with SDS of nuclei from cells labeled with ^{35}S-methionine yields labeled proteins which give virtually the same electrophoretic pattern on SDS gels as is derived from cytoplasmic proteins labeled after heat shock. This situation is in contrast to that of cells kept at 25°C. In these cells, the electrophoretic pattern of labeled proteins derived from the nucleus exhibits major differences from the pattern of labeled proteins derived from the cytoplasm. Hence an association of heat shock proteins with the chromosomes has been shown, but nothing is known of the distribution over the chromosomes of the individual proteins.

Antibodies directed against individual heat shock proteins could, by in-direct immunofluorescence (3-5), be used to reveal the distribution of the individual

proteins.

Furthermore, antibodies directed against an individual protein could be used to help identify one molecule with another, since a common molecular weight is insufficient evidence for identity.

This chapter describes the purification of the 84,000 dalton heat shock protein, the production of antibodies to that protein, and some uses of the antibodies. In particular, antibodies directed against the 84,000 dalton protein have been used to determine whether this protein is synthesized in non-heat shocked cells. Also, polytene chromosomes have been stained with the indirect immunofluorescence technique using antiserum directed against this heat shock protein.

Materials and Methods

Fly culture

Wild-type stocks of D. melanogaster were raised in mass culture as described by Mitchell and Mitchell (6). For the protein purification experiments, Oregon R and Urbana S wild-type stocks were used. For the immunoprecipitation experiments the Canton S wild-type stock was used. Salivary gland chromosome squashes were prepared from Canton S and Oregon R stocks.

SDS gel electrophoresis

SDS polyacrylamide gel electrophoresis was carried out as described in Chapter 1a, except that the stock solution of acrylamide was 30% acrylamide, 0.4% bisacrylamide (acrylamide and bisacrylamide were purchased from Bio-Rad or from Scientific Chemical Co., Inc., Huntington Beach, California), and in some cases, the separating gels were 110 mm long, rather than 90 mm long. The gels

were stained as described in Chapter 1a or below, dried, and exposed for autoradiography or fluorography (7).

Protein purification

1. Labeling. One hundred twelve 5 to 6 hr prepupae were injected with 0.6 mCi ^{35}S-methionine (New England Nuclear, specific activity ~400 Ci/mM) in 15 µl MOPS buffered medium (8) after a 20 min heat shock at 37.5°C. The prepupae were left at room temperature for 110 min to label the heat shock proteins.

2. Salt Fractionation. The injected prepupae were ground with 10 g of frozen prepupae which had been heat shocked between 4 and 8 hr after puparium formation for 20 min at 37.5°C and left at 25°C for 1 to 1.5 hr before freezing in liquid nitrogen. The prepupae together with 5 g sand were ground at near liquid nitrogen temperatures with a mortar and pestle.

The powder was then dissolved in 100 ml 0.01 M sodium phosphate buffer, 1 mM DTT (Sigma), pH 6.8, and centrifuged to remove insoluble material.

The supernatant was fractionated by precipitating proteins at 35%, 42%, 57%, 75%, and 100% saturation with ammonium sulfate. The first four fractions were precipitated by the sequential addition of 0.02 M phosphate buffer, pH 6.8, which had been saturated with ammonium sulfate, and then made 1 mM in DTT. The 75-100% fraction was precipitated by adding the salt directly to the 75% saturated solution. The precipitates were redissolved in 24 to 33 ml 0.02 M sodium phosphate, pH 6.8, 1 mM DTT. About 30 µg of protein from each fraction were run on SDS gel for analysis.

3. Hydroxylapatite Chromatography. The 42-57% and the 57-75% fractions were separately chromatographed on a hydroxylapatite (HAP) column. The 80 ml bed volume was a 1:1 mixture, by volume, of hydroxylapatite (Bio-Rad Bio-Gel HT) and celite. The column was equilibrated in 0.02 M sodium phosphate, pH 6.8, 1 mM DTT. Proteins were eluted with 0.04 M phosphate, 0.08 M phosphate, and then a gradient of 0.08 M to 0.32 M phosphate. All solutions were 1 mM in DTT. The columns were run at 4°C. The flow rate was approximately 40 ml/hr. Fractions were collected for constant intervals of time or at constant increments of volume eluted.

Portions of at least every other fraction were assayed for protein (9) and for radioactivity. To measure radioactivity, TCA precipitates of the aliquot plus 100 μg BSA (Sigma), added as carrier, were collected on filters and counted in a toluene-based scintillation fluid. Approximately 30 μg of protein from selected HAP column fractions were run on SDS polyacrylamide gels for analysis.

Slight modifications of the chromatography on HAP have been tried and these also give satisfactory results. These modifications are as follows: the column can be eluted with a gradient of 0.08 M to 0.28 M phosphate, followed by elution at 0.32 M phosphate; or phosphate buffer at pH 6.4 can be used, but the 0.08 M to 0.32 M phosphate gradient must be followed by elution with 0.32 M phosphate saturated with NaCl (at 4°C).

4. Two-dimensional Gel Electrophoresis. Two-dimensional isoelectric focusing-electrophoresis was carried out essentially as described by O'Farrell (10) except that proteins to be focused were treated with SDS (Matheson, Coleman, and Bell) before the focusing step. Ames and Nikaido (11) have shown that proteins in SDS solutions can be focused successfully.

Fractions from HAP chromatography which had been shown to contain the 84,000 dalton protein or the P protein were concentrated by lyophilization (after dialysis against volatile buffer) or by precipitation with 5% TCA (final concentration). If TCA was used, the precipitate was washed with 95% ethanol, 100% ethanol and dried.

The proteins were dissolved in SDS sample buffer (12), freshly made 60 mM in DTT, at 95°C for 15 min. At this point, the sample was treated similarly to those of Ames and Nikaido (11). To the SDS-containing solution were added 2 volumes of a solution 9% (v/v) in Triton X-100 (Sigma), which was also 2% in ampholines (LKB) in the same ratio as was present in the focusing gel. One hundred mg of ultra pure urea (Schwarz-Mann) for every 100 µl initial volume of sample buffer were then added. The sample was kept at room temperature for 2 to 4 hr before focusing.

The focusing gels were made up according to a formula similar to O'Farrell's (10) except that a total of 0.55 ml of ampholines (in 40% stock solutions) per 5.5 g urea was used, and Triton X-100 was substituted for NP-40. The Triton X-100 was used as a 10% (v/v) stock solution. To isolate the 84,000 dalton heat shock protein, stock solutions of ampholines pH 4-6, pH 5-8, and pH 7-10 were used in the ratio 3:3:5. To isolate the P protein, stock solutions of the same ampholines were used in the ratio 1:4:6. For analytical purposes, cylindrical gels 3.5 mm x 11 cm were used. For preparative purposes, gels 5 mm x 13 cm were used. The gels were pre-run at 200 V for 30 min with 50 µl or 70 µl (for analytical or preparative gels, respectively) pre-run overlay (10), 60 mM in DTT, per gel. Fifty to 500 µl sample volumes were applied to the gels and overlaid with 25 or 50 µl (for analytical or preparative gels, respectively) pre-run overlay diluted 1:2 with

H_2O. The amount of protein run on a single preparative focusing gel was from 0.1 to 0.5 mg, depending on the solubility in the SDS buffer of the particular preparation. Focusing was carried out for 12 hr (analytical gels) or 13 to 14 hr (preparative gels) at 435 V, then for 2.5 hr at 800 V.

After focusing was completed, the gels were soaked for about 30 min in sample buffer (10) either 5% in β-mercaptoethanol or 120 mM in DTT. The analytical gels were laid across the tops of 1 mm thick SDS-acrylamide slab gels, 12.5% in acrylamide, 0.17% in bisacrylamide. Preparative gels were laid across the tops of slab gels 2 mm thick. Often, for preparative purposes, the one-half or the one-third of the focusing gel containing the desired protein, was cut out, so that protein from 2 or 3 gels, respectively, could be run on one slab gel. Electrophoresis of the focused proteins through the SDS acrylamide gels was carried out at room temperature at 10-12.5 ma (analytical gels) or at 20-25 ma (preparative gels) for 6 to 9 hr.

The slab gels were stained with 0.25% Coomassie blue made up in 50% methanol (v/v) and 7.5% acetic acid (v/v) for 1 hr, and destained in 20% methanol (v/v) 7.5% acetic acid (v/v) for 1 to 3 hr (13). The spots corresponding to the 84,000 dalton protein or the P protein were cut out and subjected to electroelution immediately or frozen at -20°C until electroelution could be carried out.

To electroelute the protein, a procedure, based on the one used by Galibert et al. (15) to elute DNA from gel bands, was followed. The gel pieces were minced and placed in a 10 ml disposable pipet (Falcon), cut off at the 6 ml mark and plugged with glass wool. The protein migrated out of the gel under an electric field in Tris-glycine electrophoresis buffer (6 g Tris, 28.8 g glycine, 1 g SDS per liter, modified from Laemmli [14]) into a dialysis bag which had been fit snugly over the tip of the pipet.

The electroelution was carried out in a Canalco cylinder gel apparatus at 100-200 V, constant voltage, or 2 to 5 ma per pipet, constant current, for 12 to 18 hr at 4°C. In some cases, this was followed by electroelution in the same buffer without SDS for an additional 6 to 12 hr at about 1.5 to 2.0 ma, constant current, per pipet.

Immunization

Each of two rabbits was immunized against the 84,000 dalton protein by primary injections, given intracutaneously, of about 60 μg of protein (total per rabbit) into several sites on the back of the rabbit. These were followed by several booster injections of 50 to 100 μg each time, given in the following schedule: (1) at 6 weeks, (2) at 15 weeks, (3) at 6 months, (4) at 6 months, 3 weeks, (5) at 7 months. The first and second booster immunizations were given intracutaneously and intramuscularly, respectively. The third and fourth booster immunizations were given intracutaneously, on the back of the rabbit, and subcutaneously, in the foot pad. The last booster was given intravenously.

The 84,000 dalton protein for the primary injections was prepared by one-dimensional electrophoresis, through 2 mm thick SDS acrylamide slab gels, of hydroxylapatite fractions containing this protein. Ten sample pockets were loaded with 0.16 mg protein each. The gels were stained with Coomassie blue as described by Lazarides and Hubbard (13). The stained bands representing the 84,000 protein were cut out and homogenized in 2.7 volumes of 0.4 M sodium bicarbonate. The homogenizer was rinsed with water or 0.1% SDS, and the rinse, added to the homogenate. The final mixture was emulsified with an equal volume of Freund's Complete Adjuvant (Difco).

All of the booster injections were made with protein purified from two-dimensional gels. An alum precipitate of the eluted protein and any SDS was formed by adding $AlCl_3$ or $AlK(SO_4)_2$ to 1% (w/v) (13). The precipitate and remaining solution were emulsified with an equal volume of Freund's Complete Adjuvant. For intravenous injections, Freund's Incomplete Adjuvant (Difco) was used.

The rabbits immunized against the P protein were injected only three times: they were injected, at a separation of three weeks, with 100-200 μg of protein (total each time per rabbit) subcutaneously in the foot pad and intracutaneously on the back. The third injection was given 6 days later, intravenously to one rabbit, and intramuscularly to the other.

All of the immunizations against P protein were made with protein purified from two-dimensional gels, as described above, and precipitated with alum as described above.

Rabbits were bled 7 to 10 days after the last injection. The sera were tested for the presence of antibodies by the ring test (16) and by diffusion in Ouchterlony plates made up in barbitol buffer. Gamma globulins were prepared from normal serum and antisera by precipitation with sodium sulfate. The serum was made 18% in sodium sulfate by the addition of the salt. The precipitate was resuspended in 12% sodium sulfate in a volume 40% that of the original serum. This last step was repeated once, and the precipitate, containing the gamma globulin fraction, was redissolved at the desired concentration.

Indirect immunoprecipitation

Staphylococcus aureus strain Cowan I, fixed in formaldehyde as described

by Kessler (17), was a gift from Charles Rice or was purchased from Calbiochem (Pansorbin, Calbiochem). Within the 24 hr before each precipitation experiment, the preparation of adsorbent was washed as described (17) and resuspended in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.02% NaN_3), pH 7.4, containing 0.05% NP-40 (Shell Chemical Co.), 1 mg/ml ovalbumin (Sigma), and 5 mM methionine (17).

^{35}S-methionine labeled D. melanogaster proteins for the immunoprecipitation experiments were prepared and immunoprecipitated in two ways:

1. Lysis in Non-ionic Detergent. Eight to twelve salivary glands were labeled for 30 min in 30 μCi ^{35}S-methionine (specific activity 48 Ci/mM, New England Nuclear) in MOPS buffered medium, washed in the same medium, and lysed by freezing and thawing three times in 150 μl of the following buffer: NET/2, 0.5% NP-40, 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma).

Insoluble material was pelleted by centrifugation at 54,000 g for 90 min. The supernatant was preadsorbed with 15 μl of normal serum gamma globulin at 17 mg/ml and 45 μl of the Staphylococcal adsorbent. After 15 min at 0°C, the adsorbent was pelleted by centrifugation in the international centrifuge at 2000 rpm for 20 min. The supernatant was divided into three aliquots.

Five μl of normal serum gamma globulin (17 mg/ml), 5 μl of anti-84,000 dalton protein gamma globulin (20 mg/ml) or 5 μl of anti-P protein gamma globulin (18 mg/ml) were added to each of the aliquots. After a 15 min incubation on ice, about 6 volumes of 0.05% NP-40-NET buffer were added to each aliquot and the mixtures centrifuged. The pellets were washed once, in 0.05% NP-40-NET buffer. The protein was eluted from the pellets by heating at 95°C for 15 min in SDS sample buffer (12) in the absence of a reducing agent. The solution was then made 50 mM

in DTT, incubated at room temperature 1 hr, then made 100 mM in iodoacetamide (17). Half of each sample was analyzed by SDS polyacrylamide electrophoresis.

2. Lysis in SDS. The Staphylococcal adsorbent was washed and resuspended as described above except that the concentration of NP-40 was 0.5% rather than 0.05%. Tissue samples (8 salivary glands, 4 brains, 2 ovaries, or thoracic epithelium from 3 animals) were labeled in 30 μCi ^{35}S-methionine (specific activity 1270 Ci/mM, Amersham-Searle) in MOPS buffered medium, washed in the same medium, and lysed by incubation at 32 to 37°C for 45 to 60 min in 30 μl NET buffer made 1% in SDS.

The samples were made 0.5% in NP-40 and 0.05% in SDS by the addition of 0.53% NP-40-NET buffer containing 1 mM PMSF. The samples were cleared of insoluble material by centrifugation at 36,000 g for 60 min. The supernatants were preadsorbed by incubating with 10 μl of normal serum gamma globulin (17 mg/ml) for 15 min on ice followed by incubation with 200 μl of Staphylococcal adsorbent on ice. The adsorbent was pelleted at 4°C in the International centrifuge at 2000 rpm for 20 min. The supernatants were divided into four aliquots, one of which was again divided into two aliquots.

To each of the three aliquots were added 5 μl of normal serum gamma globulins, 5 μl of anti-84,000 dalton protein gamma globulins, or 10 μl of anti-P protein gamma globulins, respectively. To each of the half-aliquots were added 5 μl of anti-84,000 dalton protein gamma globulins or 10 μl of anti-P protein gamma globulins, respectively. After a 15 min incubation on ice, 90 μl of Staphylococcal adsorbent was added to each of the aliquots and half-aliquots. After another 15 min incubation on ice, the suspensions were diluted with 2 ml of 0.5% NP-40-NET buffer, incubated on ice 15 min more, and centrifuged.

The pellets were washed once with 1 ml 0.5% NP-40-NET buffer and then extracted with SDS sample buffer (12), 60 mM in DTT, at 85°C for 10-15 min. Sulfhydryl groups were then acetylated. Half of each sample was run on an SDS acrylamide gel.

Some of the labeled tissue lysates described above were prepared from animals that had been heat shocked. Salivary glands were dissected from late larvae or 7.5 hr prepupae, and brains were dissected from late larvae, immediately after a heat shock of 37.5°C for 20 min. Ovaries were dissected from 4-5 day adults immediately after a heat shock of 37.5°C for 40 min. Thoracic epithelial tissue was dissected from pupae 7 hr after a heat shock of 40.2°C for 40 min. The pupae were heat shocked at 36 hr after puparium formation.

Indirect immunofluorescence

Chromosomes for binding studies with antisera were prepared by acid fixation of salivary glands as described by Mitchell and Lipps (2) or by the formaldehyde fixation technique described by Silver and Elgin (3), except that, in the case of formaldehyde-fixed glands, no postfixation in formaldehyde was done. In both preparation techniques, the slides were washed in 95% alcohol for 30 min to 1 hr immediately after removing the coverslip (4). Incubations with antisera were carried out essentially as described by Silver and Elgin (18).

In the experiments using acid-fixed chromosomes from isolated nuclei, dried slides were wet in PBS (10 mM sodium phosphate, 150 mM NaCl), pH 7.5, for about 1 hr, incubated with 18 mg/ml bovine gamma globulin (Pentex) for 30 min, washed with PBS, and incubated with normal serum gamma globulins, 18 mg/ml, or antiserum gamma globulins, 18 mg/ml, for 30 min. After washing in PBS, the

chromosome squashes were treated for 30 min with fluorescein-conjugated goat-anti-rabbit gamma globulins (Antibodies, Inc.) diluted 20 times. After further washing, coverslips were mounted and the slides stored at -20°C or immediately viewed in the fluorescence microscope.

In experiments using the formaldehyde-fixed chromosomes, the slides were either used immediately after the wash in 95% ethanol or stored at -20°C in glycerol-TBS (2:1; v/v). TBS is 10 mM Tris, 0.85% NaCl, pH 7.15 at 25°C. The slides were soaked in TBS for about 2 hr, incubated in glycine-Tris buffer (0.5 M glycine, pH 8.3 at 25°C) for 30 min at 4°C, rinsed in TBS, and incubated with preimmune serum or antiserum diluted 20 times in TBS. The diluted sera were made 2 mg/ml in bovine gamma globulin. After washing about 15 min in TBS, the chromosome spreads were incubated with a 40 times dilution of fluorescent goat anti-rabbit gamma globulins (F/P = 2.6, Antibodies, Inc.). After washing in TBS for about 30 min, coverslips were mounted in a drop of 9:1 glycerol:1 M Tris (pH 7.6 at 25°C) (18).

Slides were photographed under incident ultraviolet illumination on a Zeiss fluorescence microscope (courtesy of W. Dreyer) using a 40X objective. Exposures of 15 sec were made on Kodak Tri-X film. The film was developed with Diafine (Kodak). All fluorescent prints were made on Ilford contrast 5 paper using the same exposure for different pictures.

To photograph the banding patterns of the chromosomes after the fluorescence pictures had been taken, the slides were immersed in 95% ethanol until the coverslips came off. The slides were then air dried and stained with 1% orcein in 1:1 lactic acid:acetic acid (85% lactic acid, glacial acetic acid). Coverslips

were mounted with Paramount and photographed, using phase contrast optics, at the same magnification as used for the fluorescence pictures.

Results

Purification of the 84,000 dalton and P proteins

Proteins from D. melanogaster pupae which had been heat shocked at 37.5°C for 20 min were fractionated by precipitation with ammonium sulfate, followed by chromatography on hydroxylapatite (HAP). ^{35}S-methionine labeled protein from heat shocked prepupae was included as a tracer to follow the purification of the individual heat shock proteins. Figure 1 shows the protein fractions precipitated in 0-35%, 35-42%, 42-57%, 57-75%, and 75-100% saturated solutions of ammonium sulfate. Table 1 lists the amounts of protein and of radioactivity recovered in each fraction. The enrichment of individual labeled proteins over others in the various fractions can be noted by visual inspection of the fluorogram in Figure 1. Table 1 also lists the percentage of radioactivity in bands corresponding to the 84,000, the 70,000, and the 27,000 dalton proteins in the first four fractions. Since the amount of radioactivity in the last fraction is relatively low, this fraction will be disregarded in further discussions. The values in Table 1 were obtained by measuring the peak areas versus the total areas in densitometer scans of the fluorogram. These data show that the enrichment for the 84,000 dalton protein relative to the 70,000 dalton protein is greater in the 42-57% salt fraction than in any other. Also, the 27,000 dalton protein is enriched, with respect to the 70,000 dalton protein, in fractions 35-42% and 42-57%. The 70,000 dalton protein is enriched with respect to the others in the 57-75% fraction. Thus the

Figure 1. Ammonium sulfate fractionation of proteins from heat shocked prepupae. One hundred ten prepupae labeled with ^{35}S-methionine for 1 hr after heat shock were ground with 10 g frozen heat shocked prepupae. After addition of 100 ml phosphate buffer, the proteins were fractionated by stepwise precipitation with ammonium sulfate. About 30 μg of protein from each of five ammonium sulfate fractions were run through an SDS electrophoresis gel. The gel was stained (left) and exposed for fluorography (right). The number above each lane indicates the percentage of saturation with ammonium sulfate at which the proteins of each fraction precipitated. The positions to which the heat shock proteins migrated are indicated at the right. Left, Coomassie blue stained gels; right, fluorograph.

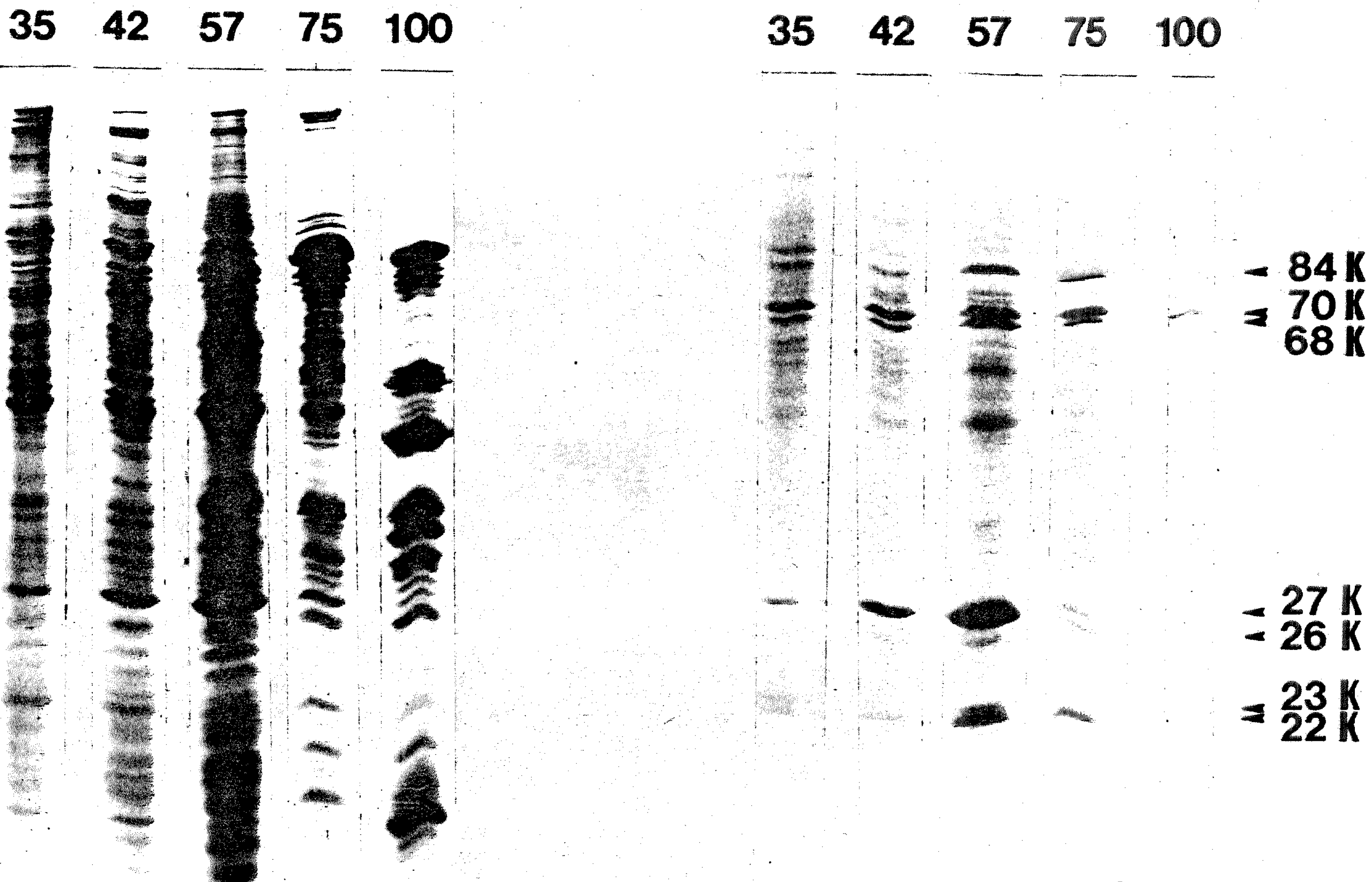

35
42
57
75
100
35
42
57
75
100
84 K
70 K
68 K
27 K
26 K
23 K
22 K

Table 1

Fraction	Total protein (mg)	Total radioactivity (cpm)	84,000 dalton protein		70,000 dalton protein		27,000 dalton P protein	
			Percentage of total radioactivity	Specific activity* (cpm/mg)	Percentage of total radioactivity	Specific activity (cpm/mg)	Percentage of total radioactivity	Specific activity (cpm/mg)
Ammonium sulfate								
0-35%	65	4.68×10^6	2.4%	1.7×10^3	16.3%	11.7×10^3	2.5%	1.8×10^3
35-42%	45	2.33×10^6	4.0%	2.1×10^3	18%	9.3×10^3	19.8%	10.2×10^3
42-57%	160	8.41×10^6	3.6%	1.9×10^3	9.1%	4.8×10^3	42%	22.1×10^3
57-75%	90	2.50×10^6	5.7%	1.6×10^3	37.3%	10.4×10^3	2.8%	0.7×10^3
75-100%	40	0.47×10^6	-	-	-	-	-	-
HAP chromatography of 42-57% ammonium sulfate fraction								
34							71%	162×10^3
88					40%	16.8×10^3		
98					47%	22.6×10^3		
130			44%	19.7×10^3				
HAP chromatography of 55-75% ammonium sulfate fraction								
68					43%	17.7×10^3		
94					31%	7.5×10^3		
110			45%	12.1×10^3				

*Specific activity in this table is defined as the radioactivity in a given band per total amount of protein in the fraction.

42-57% fraction would be chosen for purifying the 84,000 and 27,000 dalton proteins, and the 57-75% fraction, for purifying the 70,000 dalton protein.

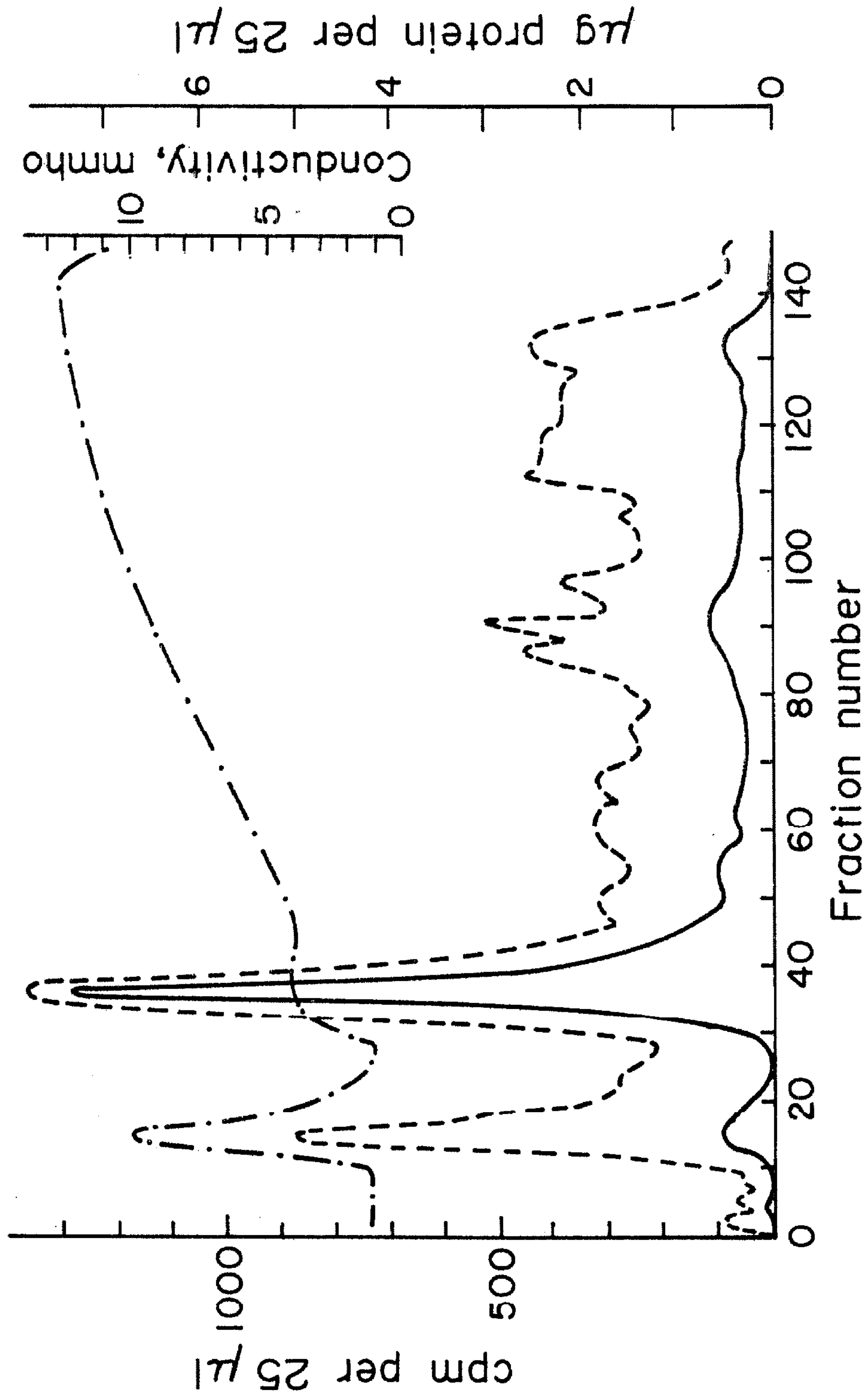
Specific activities, calculated as the radioactivity in a given band, or protein, per total amount of protein, are also listed in Table 1. From the point of view of specific activities, the 84,000 dalton protein is not enriched significantly in any fraction, but since as large an amount of pure protein as possible is desired for immunization purposes, it seems that the best fraction from which to purify the protein is the 42-57% fraction. The 27,000 dalton protein, on the other hand, is very much purified in the 42-57% fraction (Table 1).

The radioactivity in the band representing the 27,000 dalton protein does not entirely represent the 27,000 dalton heat shock protein(s). As will be discussed later, a large part of this radioactivity is due to protein synthesized at a high rate normally in non-heat shocked cells, and in this case, the synthesis persists (or has resumed) after heat shock. This protein has been named P (for plentiful), and the figures in Tables 1 and 2 are calculated for total radioactivity in the band which migrates as a 27,000 dalton protein.

The 42-57% salt fraction was initially selected to chromatograph on hydroxylapatite because this fraction had the most total radioactivity (Table 1). Figure 2 shows the radioactivity and protein elution profiles from a hydroxylapatite column which was 50% by volume celite (to improve the flow rate). Selected fractions were analyzed by SDS gel electrophoresis. The Coomassie blue stained pattern and the fluorogram of this gel are shown in Figure 3. One can see that most of the labeled proteins are fractionated by chromatography on this adsorbent.

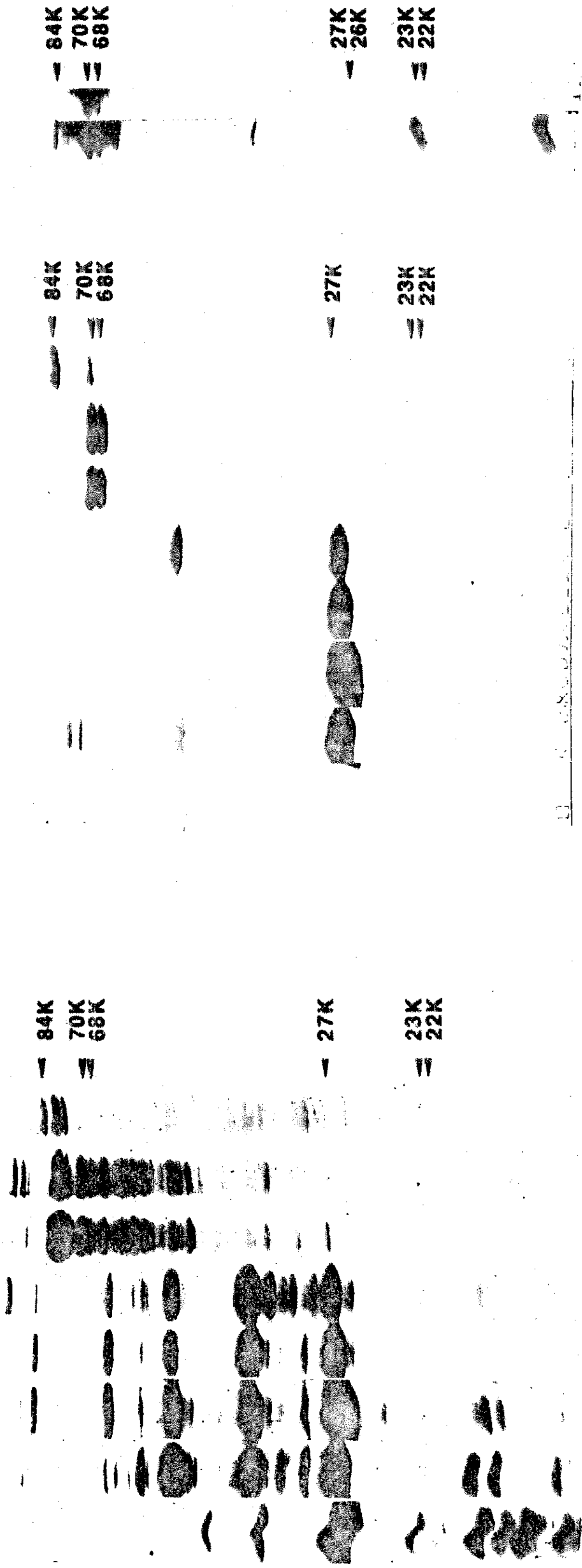
Again, densitometer scans of the fluorograms were made and percentage of radioactivity in various bands was measured. The specific activities of the

Figure 2. Hydroxylapatite chromatography of the 42-57% ammonium sulfate fraction. One hundred ten mg of the protein which precipitated between 42 and 57% saturation with ammonium sulfate were applied to an 80 ml column of a 1:1 (by volume) mixture of hydroxylapatite and celite. The proteins were eluted with increasing concentrations of sodium phosphate buffer. Fraction volumes are between 4.5 ml and 6.0 ml. The continuous line represents radioactivity; the dashed line, protein content; and the dotted-dashed line, conductivity.



cpm per 25 μl
1000
500
0
20
40
60
80
100
120
140
Fraction number
Conductivity, mmho
10
5
0
μg protein per 25 μl
6
4
2
0

Figure 3. SDS gel electrophoresis of selected eluted fractions from the hydroxylapatite column. About 30 μg of protein from the fraction designated above each lane were run on the gel. An exception is the material in fraction 130; because of solubilization difficulties, only 10 μg were run in this lane. The fraction numbers above each lane correspond to those in Figure 2. The positions to which the heat shock proteins migrated are indicated at the right. The lanes marked "SG" represent two different exposures of a salivary gland sample labeled with ^{35}S-methionine after heat shock.


SG SG
84K
70K
68K
27K
26K
23K
22K
14 34 38 44 55 86 96 130
84K
70K
68K
27K
23K
22K
14 34 38 44 55 86 96 130
84K
70K
68K
27K
23K
22K

84,000, 70,000, and 27,000 dalton proteins were measured and are listed in Table 1. Comparison with the specific activities of the 42-57% ammonium sulfate fractions gives purification values of 10.4-fold for the 84,000 dalton protein in hydroxylapatite fraction 130, 4.7-fold for the 70,000 dalton protein in fraction 98, and 7.3-fold for the 27,000 dalton protein in fraction 34.

The Coomassie blue stained pattern of fraction 130 (Fig. 3) shows a band which corresponds exactly to the band representing the 84,000 dalton protein in the fluorogram. The outline of the band is exactly the same, suggesting that this band represents only the 84,000 dalton protein. Two-dimensional isoelectric focusing-electrophoresis gels show no other spots at this molecular weight, although this is not conclusive evidence that the protein in the band is homogeneous. Some proteins will not enter a focusing gel, one reason being that the isoelectric pH of the protein may be out of the range of the focusing gel.

The Coomassie blue stained lane representing the protein in fraction 130 was scanned on the densitometer. Not all proteins are stained with the same efficiency by Coomassie blue, but at least a rough estimate of the percentage of the 84,000 dalton protein in fraction 130 can be made. The result of the densitometric measurement is that 8% of the protein in the fraction is the 84,000 dalton protein. By using the specific activity (240 cpm/μg) of the protein in this band, the concentration of the 84,000 dalton protein in the ammonium sulfate fractions can be calculated. These values are listed in Table 2.

The 27,000 dalton-P protein also is detectable as a Coomassie blue staining band. This band, which is identical in width and general appearance to the corresponding fluorogram band, accounts for 38% of the Coomassie blue stained material in hydroxylapatite fraction 34. The specific activity of this protein (i.e.,

Table 2

Fraction	Percentage of total protein		
	84,000 dalton protein	70,000 dalton protein	27,000 dalton P protein
Ammonium sulfate			
0-35%	1.0%	6%	0.6%
35-42%	1.2%	5%	3.5%
42-57%	1.2%	2%	7.5%
57-75%	0.9%	5%	0.3%
HAP chromatography of 42-57% ammonium sulfate fraction			
34			38%
88		6%	
98		8%	
130	8%	2%	
HAP chromatography of 57-75% ammonium sulfate fraction			
68		11%	
94		3%	
110	5%		

Some of the values in the table were measured. Other values were calculated using the following values for specific activity: 240 cpm/μg for the 84,000 dalton protein, 280 cpm/μg for the 70,000 dalton protein, 420 cpm/μg for the 27,000 dalton P protein. Specific activities in this case are defined as the radioactivity in a given protein per amount of that protein.

the protein only in this band) is 420 cpm/μg. The concentrations of this protein in the ammonium sulfate fractions are listed in Table 2.

It is not clear in hydroxylapatite fraction 98 (Fig. 3) that one Coomassie blue staining band corresponds exactly to the band on the fluorogram representing the 70,000 dalton protein. The Coomassie stained band may contain protein other than the radioactive 70,000 dalton protein. However, fraction 130 (Fig. 3) has a faint Coomassie blue staining band at 70,000 daltons which seems to correspond exactly to a band on the fluorogram. This band accounts for 2% of the Coomassie blue staining material in fraction 130 and, if the protein is homogeneous, it has a specific activity of 280 cpm/μg. Based on this value for the specific activity, the concentrations of this protein in hydroxylapatite fractions 88 and 98 and in the ammonium sulfate fractions have been calculated (Table 2).

Since the 84,000 and 27,000 dalton proteins represented relatively large proportions of the fractions they were in, and since these proteins could be fairly easily purified by gel electrophoresis, they were used for the production of antibodies.

To prepare the 84,000 dalton protein for the primary immunization of rabbits, several hydroxylapatite fractions from the elution peak around fraction 130 were run on a preparative scale (2 mm thick) one-dimensional SDS polyacrylamide gel. After staining the gel with Coomassie blue, the bands representing the 84,000 dalton protein were cut out. As described above, the 84,000 dalton protein in hydroxylapatite fraction 130 migrates as a band clearly separated from others in the same sample.

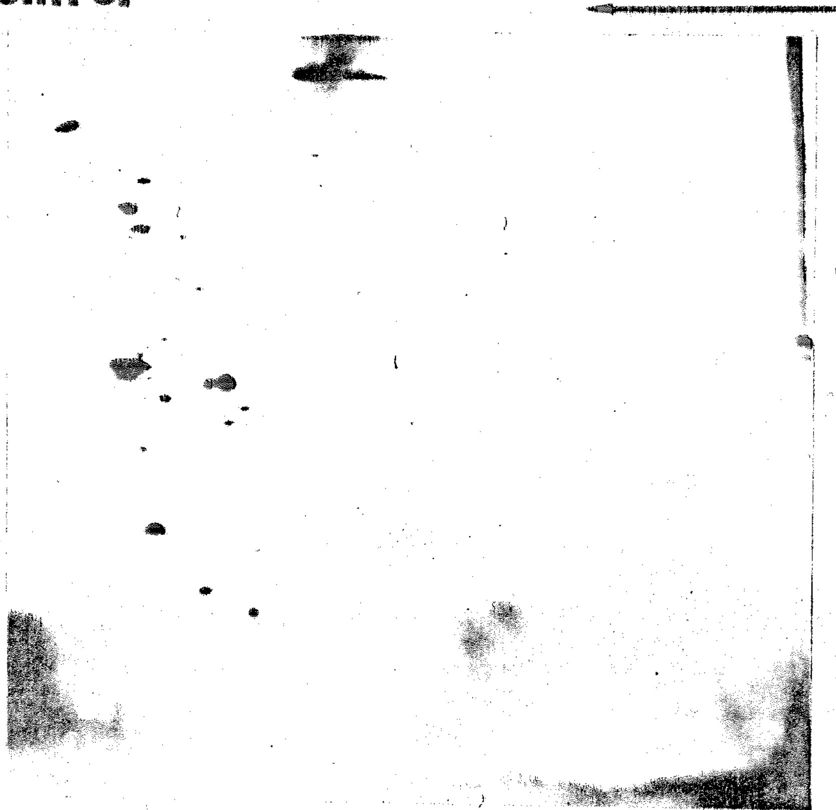
The 57-75% ammonium sulfate fraction was also chromatographed on hydroxylapatite. When fractions containing the 84,000 dalton protein were run

on SDS gels, the 84,000 dalton protein was not so well resolved from other proteins migrating near it. Since these hydroxylapatite fractions were also used to prepare the protein, and in order to assure purity of the antigen, the 84,000 dalton protein was thereafter isolated from preparative scale two-dimensional gels. It is generally accepted that the antigen for primary immunization need not be as pure as the antigen used for secondary immunizations.

The separation of the heat shock proteins on an analytical two-dimensional gel is shown in Figure 4. The proteins were derived from ^{35}S-methionine labeled salivary glands from prepupae. The approximate isoelectric points of the heat shock proteins are as follows: 84,000 dalton protein, pH 5.5; 70,000 dalton protein(s), pH 6.0-6.2; 68,000 dalton protein, pH 6.2-6.3; 27,000, 26,000 proteins, pH 7.5-7.6; and 23,000, 22,000 dalton proteins, pH 5.8-6.0. The 70,000 dalton protein separates as at least two spots on gels in which a less steep gradient is used.

The 84,000 dalton protein-containing fractions were run on isoelectric focusing gels made up with a slightly different mixture of ampholines from the mixture used on the gel represented in Figure 4. This adjustment was made to give better separation of proteins in the region of the 84,000 dalton protein. Figure 5 shows part of an analytical scale two-dimensional gel of hydroxylapatite fraction 130. The large Coomassie blue stained spot, which corresponds exactly to the 84,000 dalton protein spot on the fluorogram, is one of the major stained spots on the gel and the corresponding spot on the fluorogram is the major spot on the fluorogram. The protein in this spot migrates to the same isoelectric point as the 84,000 dalton heat shock protein from salivary gland samples run on an identical gel. There are other radioactively labeled proteins in the vicinity of the 84,000

Figure 4. Autoradiograms of two-dimensional isoelectric focusing-electrophoresis gel of prepupal salivary glands. Five salivary glands from prepupae kept at 25°C (Control) and six salivary glands from prepupae heat shocked at 37.5°C for 20 min (Heat shocked) were dissected and labeled with ^{35}S-methionine for 20 min. After precipitation with 10% TCA and washing with ethanol, the proteins were extracted into SDS sample buffer at 95°C for 10-15 min. Two-dimensional focusing-electrophoresis was carried out as described in Materials and Methods. Separation by focusing is in the horizontal direction (arrow), with the acidic end at the left; separation by SDS electrophoresis is from top to bottom (arrow). The pH gradient was formed with a 1:4:6 mixture of the following stock (40% w/v) ampholine solutions: pH 4-6, pH 5-8, pH 7-10. The positions to which the heat shock proteins migrated are indicated, by their respective molecular weights given in thousands of daltons, to the left of the lower gel.


Control

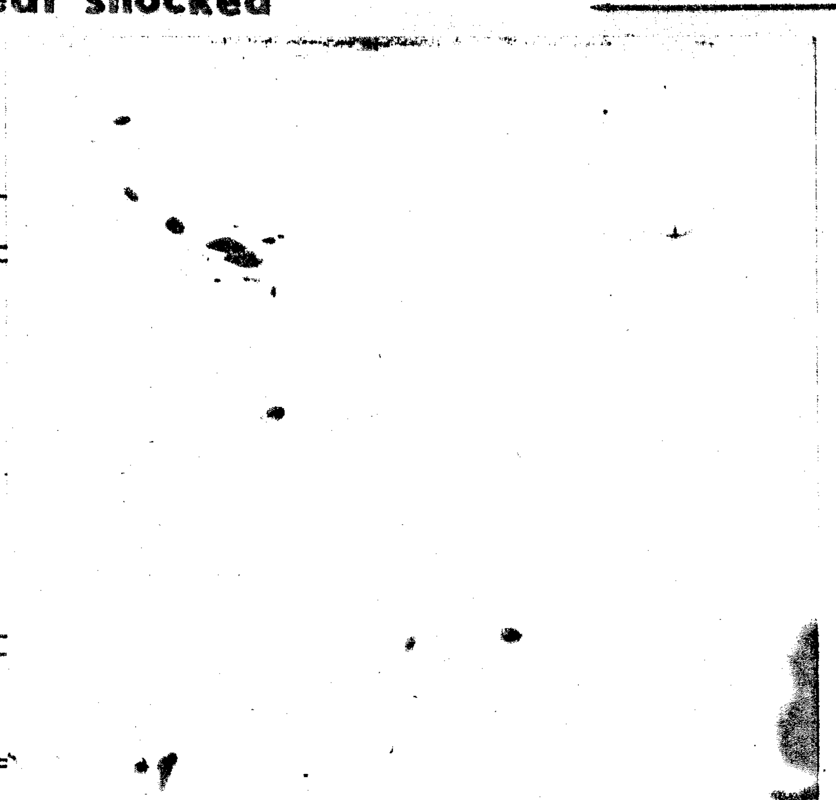
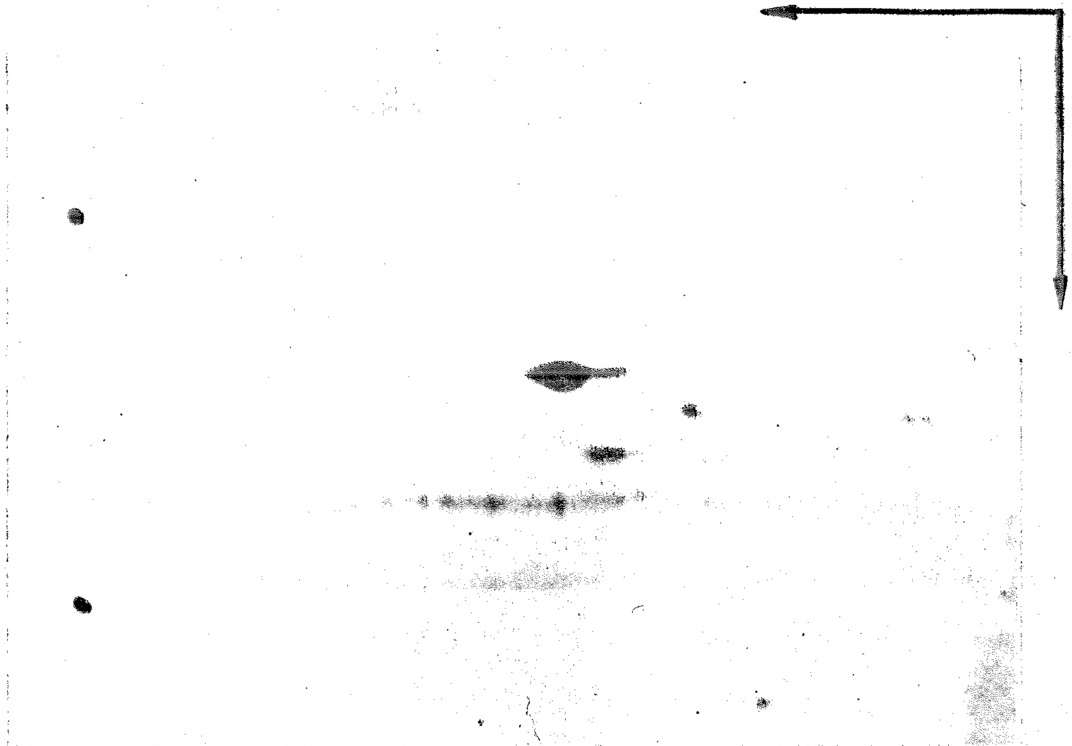
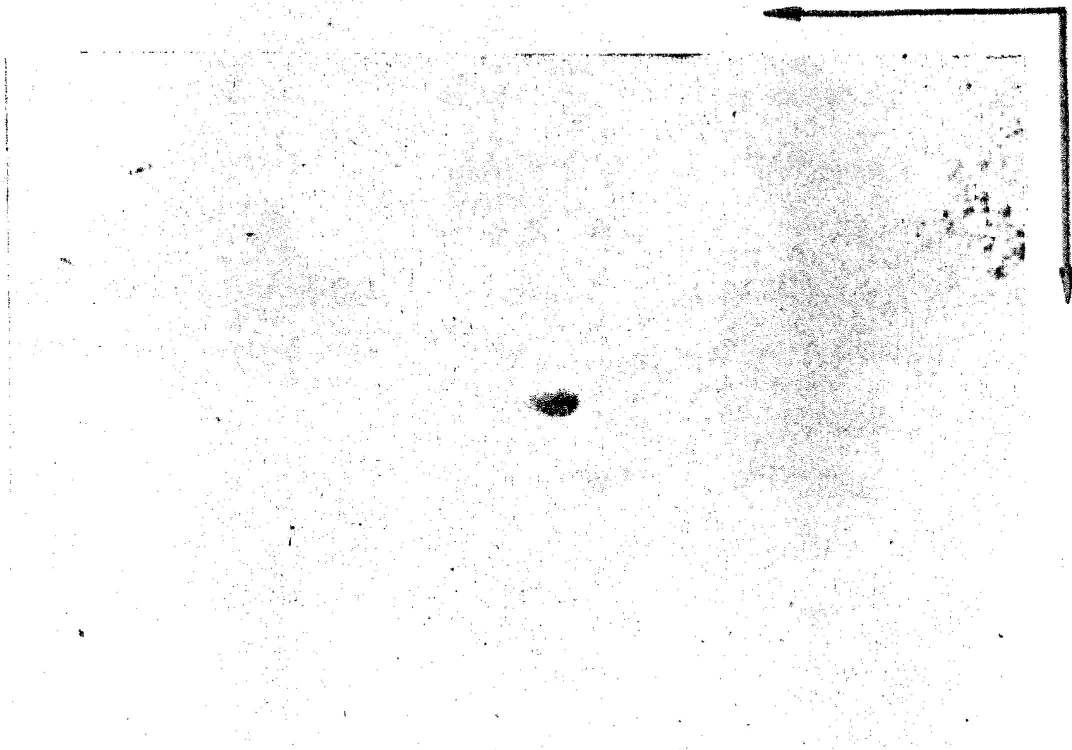

Heat shocked
84
70
68
27
26
23
22

Figure 5. Two-dimensional isoelectric focusing-electrophoresis of a partially purified preparation of the 84,000 dalton heat shock protein. A portion of fraction 130 (see Figs. 2, 3) from hydroxylapatite chromatography of the 42-57% ammonium sulfate fraction was run on a two-dimensional isoelectric focusing-electrophoresis gel; the gel was stained, and exposed for fluorography. The pH gradient was formed with a 3:3:5 mixture of the following stock (40% w/v) ampholine solutions: pH 4-6, pH 5-8, pH 7-10. Focusing is in the horizontal direction (arrow). SDS electrophoresis was from top to bottom (arrow). Shown is the upper, acidic quadrant of the gel, with the more acidic side at the left. The two spots at the left are marks that were made on the gel with a mixture of India ink and ^{35}S-methionine. Upper frame, Coomassie stained pattern; lower frame, fluorograph.





dalton protein, including the 70,000 and 68,000 dalton proteins, but these are well separated from the 84,000 dalton protein.

For two-dimensional gel preparation of the 84,000 dalton protein, 0.1 to 0.5 mg of total protein, from fractions of hydroxylapatite-chromatographed ammonium sulfate fractions, was run on each focusing gel. The focusing gel, or a section of the focusing gel containing the 84,000 dalton protein, was then run through a 2 mm thick SDS slab gel. The gel was stained, and the 84,000 dalton protein spot, cut from the gel with a scalpel. The remainder of the gel was treated and exposed for fluorography to check that the correct spot had been cut out. If there was any doubt that the spot was the correct one, the protein was not used for immunization until the fluorogram results had been obtained. Almost always in the fluorogram a halo of grains surrounding the cut out spot was seen, indicating that the protein cut out was the correct one.

The 27,000 dalton-P protein in hydroxylapatite fractions 34 to 38 exhibits a variable mobility on gels with respect to the 27,000 and 26,000 dalton heat shock proteins. Sometimes it migrates within the range of the 27,000 and 26,000 dalton proteins, and sometimes it migrates faster than the 26,000 dalton protein. This variation depends at least in part on the concentration of acrylamide in the gel and on the length of time electrophoresis is carried out. When fractions between 34 and 38 were run on two-dimensional gels, the P protein was observed to have an isoelectric point of about pH 7.1, which is slightly different from the isoelectric pH of either the 27,000 or the 26,000 dalton protein. No Coomassie stained spots corresponding to the 27,000 and 26,000 dalton heat shock proteins could be detected on these gels.

All of the P protein for immunization was purified from two-dimensional gels. Fluorograms of the remainder of the cut out gels were not made, since there was never any doubt about the identity of this spot. The Coomassie blue stained spots were by far the major spots on the gels and were also much larger than the spots corresponding to the 84,000 dalton protein. Usually 70 μg of the protein run on one focusing gel was P protein.

Indirect immunoprecipitation of the 84,000 dalton and P proteins from cell lysates

Antibodies were reacted with lysates of salivary gland cells to ascertain that the antibodies were indeed specific for the intended antigens. Salivary gland lysates from larvae and from prepupae, from animals kept at 25°C and from animals exposed to 37.5°C for 20 min were prepared in buffer that was 0.5% in NP-40. Salivary gland cells do not lyse readily in a hypotonic medium containing NP-40, so lysis was effected by repeated freezing and thawing. In another experiment, mechanical disruption of the cells with a glass rod was used. This technique is also effective, although less likely to give reproducible results.

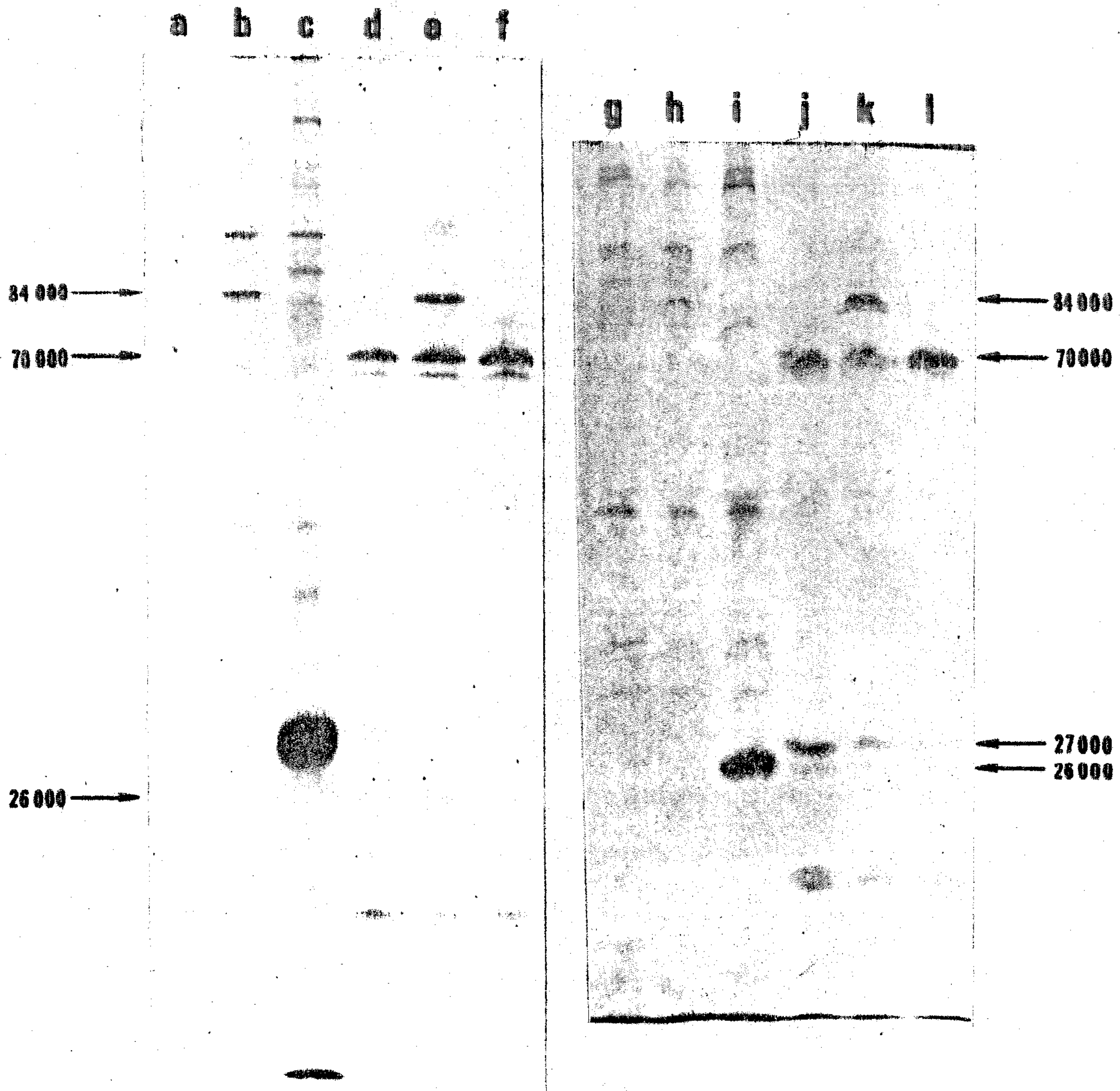
Preadsorbed cell lysates were treated with gamma globulins from normal serum or antiserum. The immune complexes were precipitated by the addition of Staphylococcal adsorbent.

As can be seen in Figure 6, gamma globulins from antiserum directed against the 84,000 dalton protein cause the specific precipitation of that protein from the NP-40 lysates of heat shocked cells. Moreover, it appears that an 84,000 dalton protein is also precipitated from lysates of non-heat shocked cells.

Gamma globulins from antiserum directed against the P protein cause the precipitation of labeled P from non-heat shocked cells, but not from heat

Figure 6. Indirect immunoprecipitation of labeled proteins from lysates of heat shocked and non-heat shocked cells. NP-40 lysates were prepared from ^{35}S-methionine labeled salivary glands of larvae (a-f) and prepupae (g-l), from heat shocked glands (d-f, j-l) and from non-heat shocked glands (a-c, g-i). The lysates were preadsorbed with normal serum gamma globulin and Staphylococcal adsorbent. To aliquots of each lysate (of 8 to 12 glands) were added 85 μg of normal serum gamma globulin (a, d, g, j), 90 μg of gamma globulin from anti-84,000 dalton protein serum (b, e, h, k), or 200 μg of gamma globulin from anti-P serum (c, f, i, l). Sixty μl of Staphylococcal adsorbent (10%, w/v) were added to each aliquot.

Precipitates were washed, extracted with SDS sample buffer, and the extracts run on SDS gels either within one day of the precipitation (g-l) or three weeks after precipitation (a-f). Gels were dried and exposed for autoradiography. The positions to which some proteins of known molecular weight migrated are indicated at the sides of the gels.


a
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70000
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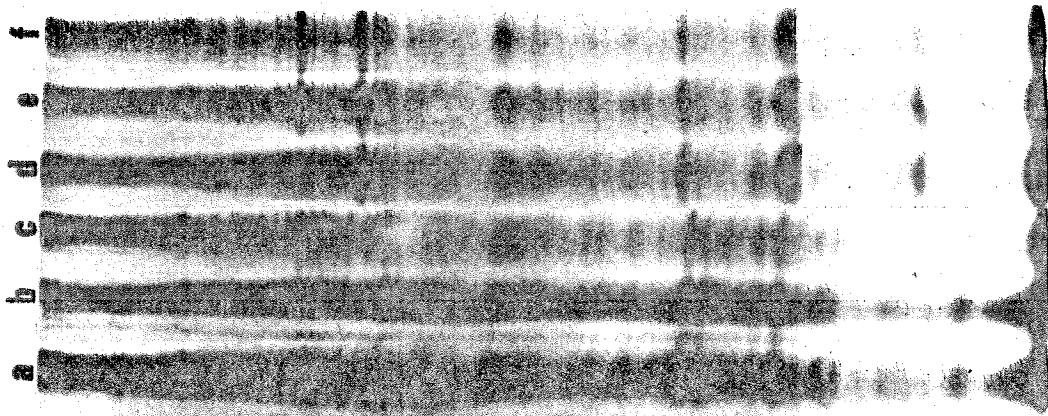
26000

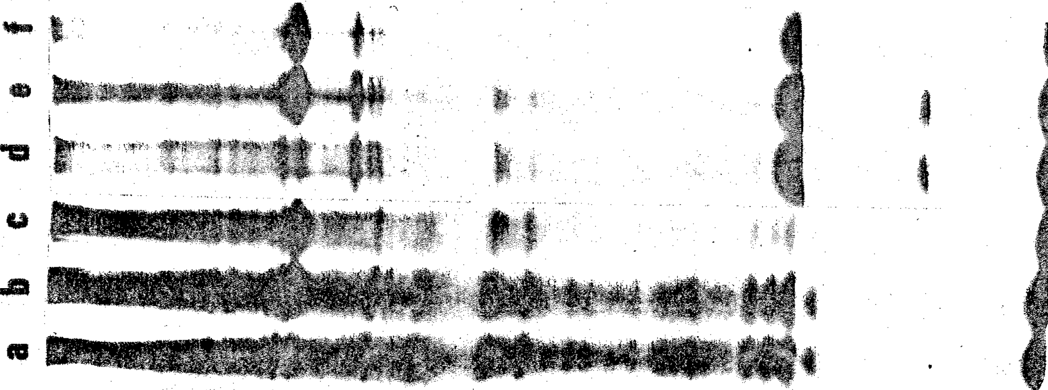
shocked cells. The synthesis of P protein in heat shocked cells is variable, and thus in pulse-labeled heat shocked cells, labeled P protein will not always be precipitated. Furthermore, from the data presented in Table 2, P protein is probably in high concentration in the whole animal. Thus it is possible that when P protein is synthesized at low rates in heat shocked cells, the specific activity of P protein may be so low, relative to other labeled proteins, that if antibody is not in excess of antigen, the amount of radioactivity precipitated may be too low to see. The protein specifically precipitated with anti-P protein gamma globulins from non-heat shocked cells migrates with a mobility corresponding to 25,000 to 27,000 daltons if run immediately after (within 24 hr) after precipitation, and the protein derived from larval tissues and from prepupal tissue has the same mobility. However, in a sample that is run 3 weeks after precipitation and elution from the adsorbent (Fig. 6), the mobility is decreased.

The 84,000 protein is precipitated from salivary gland lysates treated with SDS as well (data not shown). Lysis in SDS seems to be the optimal way to extract as many proteins as possible in high yield from any tissue. It is known that all of the heat shock proteins can be extracted from tissue directly into an SDS sample buffer (1% SDS, 5-10% sucrose, 10 mM Tris-HCl (pH 8), 10 mM EDTA, 40 mM DTT) for the following tissues: adult ovaries (Petersen, Moller, and Mitchell, personal communication), pupal thoracic epithelial tissue (Chapter 1b), larval brains, and larval salivary glands (G. Moller, personal communication). These three tissues were lysed in SDS-NET buffer and tested with the gamma globulin fraction from antiserum directed against the 84,000 dalton protein. Lysates were prepared from heat shocked and non-heat shocked animals. The results are shown in Figure 7. Lanes e in Figure 7 show that the 84,000 dalton protein is indeed

Figure 7. Indirect immunoprecipitation of the 84,000 dalton heat shock protein from different tissues. SDS lysates were prepared from ^{35}S-methionine labeled larval brains (from 4 animals), pupal thoracic epithelial tissue (from 3 animals), and adult ovaries (from 2 animals), from heat shocked (d-f) and non-heat shocked (a-c) animals. The lanes marked (a) and (d) represent aliquots of the preadsorbed lysate (see Materials and Methods) to which were added 85 μg of normal serum gamma globulin; the lanes marked (b) and (e) represent aliquots of the pretreated lysate to which were added 90 μg of gamma globulin from anti-84,000 dalton protein serum; the lanes marked (c) and (f) represent half aliquots of the pretreated lysates to which were added 90 μg of gamma globulin from anti-84,000 dalton protein serum. Ninety μl of Staphylococcal adsorbent (10%, w/v) were added to each aliquot.

Precipitates were washed, extracted with SDS buffer and the extracts run on SDS polyacrylamide gels. The brain samples, thoracic epithelial samples, and ovary samples were run on different gels. The gels were treated for fluorography, dried, and exposed 30 days (brains) or 10 days (thoracic epithalial tissue, ovaries) to X-ray film. The position to which the 84,000 and 70,000 dalton heat shock proteins migrated in the gels is indicated at the left.


OVARIES
a b c d e f


THORACIC EPITHELIUM
a b c d e f

BRAINS
a b c d e f
84K—
70K—

precipitated with gamma globulins after denaturation of the antigen with SDS. Lanes b in Figure 7 show that the same antigen can be precipitated from non-heat shocked cells from thoracic epithelial tissue and from ovaries. There is also a very faint band at 84,000 daltons in the brain samples. Lanes f and c show the results of the same immunoprecipitation tests as are shown in lanes e and b respectively, but the antibody to antigen ratio was higher in the lysates represented in lanes f and c.

Aliquots of the same lysates presented in Figure 7 and aliquots of a salivary gland lysate were tested with the gamma globulin fraction from anti-P protein serum. No protein in the range of 27,000 daltons was precipitated from any tissue except prepupal salivary glands, from animals kept at 25°C, in which case only very little labeled P protein could be detected. These gamma globulins, however, precipitate a 90,000 dalton protein from brains, from both heat shocked and non-heat shocked animals (data not shown). No experiments to analyze this phenomenon further have been carried out.

Indirect immunofluorescence of polytene chromosomes

Salivary gland chromosome squashes were treated with antisera or immune gamma globulins or with diluted normal serum or non-immune gamma globulins, and subsequently, with goat anti-rabbit immunoglobulin gamma globulins. When squashes were prepared without prior formaldehyde fixation, that is, by the method of Mitchell and Lipps (2), some staining of the chromosomes using the anti-P protein gamma globulin fraction was observed. This staining seemed to be restricted to interband regions and to the outline of the chromosomes. Staining was also observed when normal serum gamma globulin was used, but the staining was more

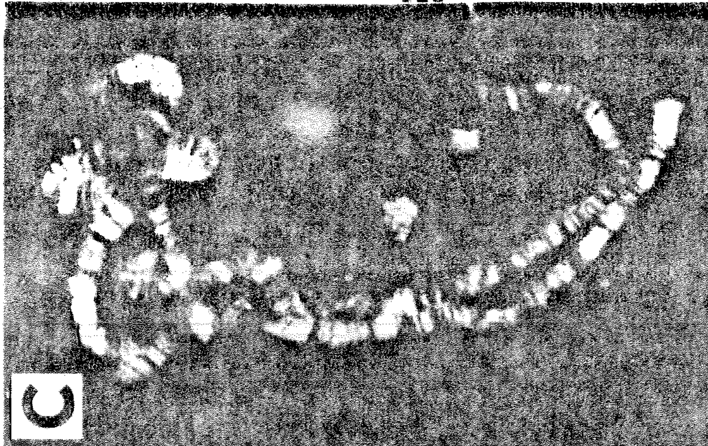
diffuse over the chromosomes. Very little staining was observed when the chromosomes were treated with anti-84,000 dalton protein serum gamma globulins. In all of these experiments, rather high concentrations of gamma globulins were used, and the intensity of the fluorescence staining was rather low.

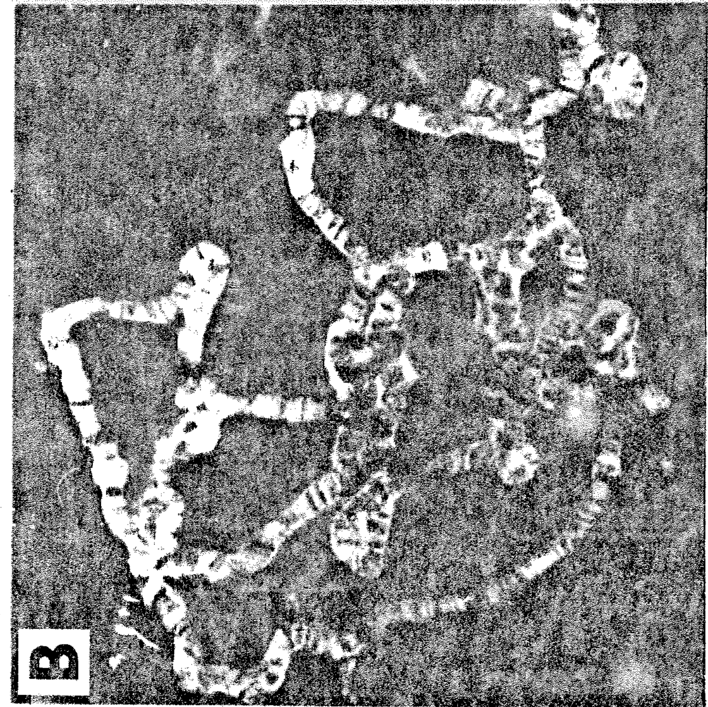
In contrast, when the chromosomes were fixed in formaldehyde, essentially as described by Silver and Elgin (3), much more staining was observed. When antiserum directed against the 84,000 dalton protein, diluted 20 times, was used to treat chromosomes prepared from larvae kept at 25°C, very bright fluorescence staining in a banded and irregular pattern was observed (Fig. 8). All of the chromosomes on the slide were stained to approximately the same intensity. This result was reproducible. The fluorescence observed when antiserum was used was much greater than that observed when preimmune serum was used (Fig. 8). However, the little staining that was observed using normal serum was rather restricted. Less than 20 fluorescent bands per chromosome squash were observed. It was not determined whether these stained regions were the same from squash to squash.

The squashes which were photographed for fluorescent staining were also stained with orcein. When the fluorescent pattern was compared with the stained DNA pattern, it became apparent that the fluorescein-labeled antibodies were situated mostly at the interband regions of the polytene chromosomes. Figure 9 shows an example of this correspondence of fluorescent bands with interband regions. The chromosome spread here was the same as the one depicted in Figure 8C.

When chromosomes from heat shocked cells were treated with the anti-84,000 dalton protein antiserum, the immunofluorescent staining was of extremely variable intensity. Some spreads were stained as much as spreads depicted in

Figure 8. Indirect immunofluorescence staining of polytene chromosomes using antiserum directed against the 84,000 dalton protein. Salivary gland chromosomes from larvae kept at 25°C were fixed by formaldehyde and stained using preimmune serum diluted 20 times (A) or antiserum diluted 20 times (B), (C). (B) and (C) are from the same slide.


C


B

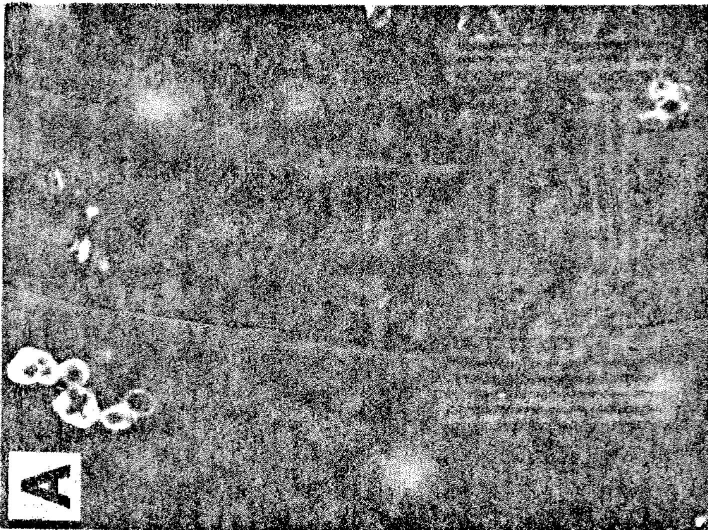
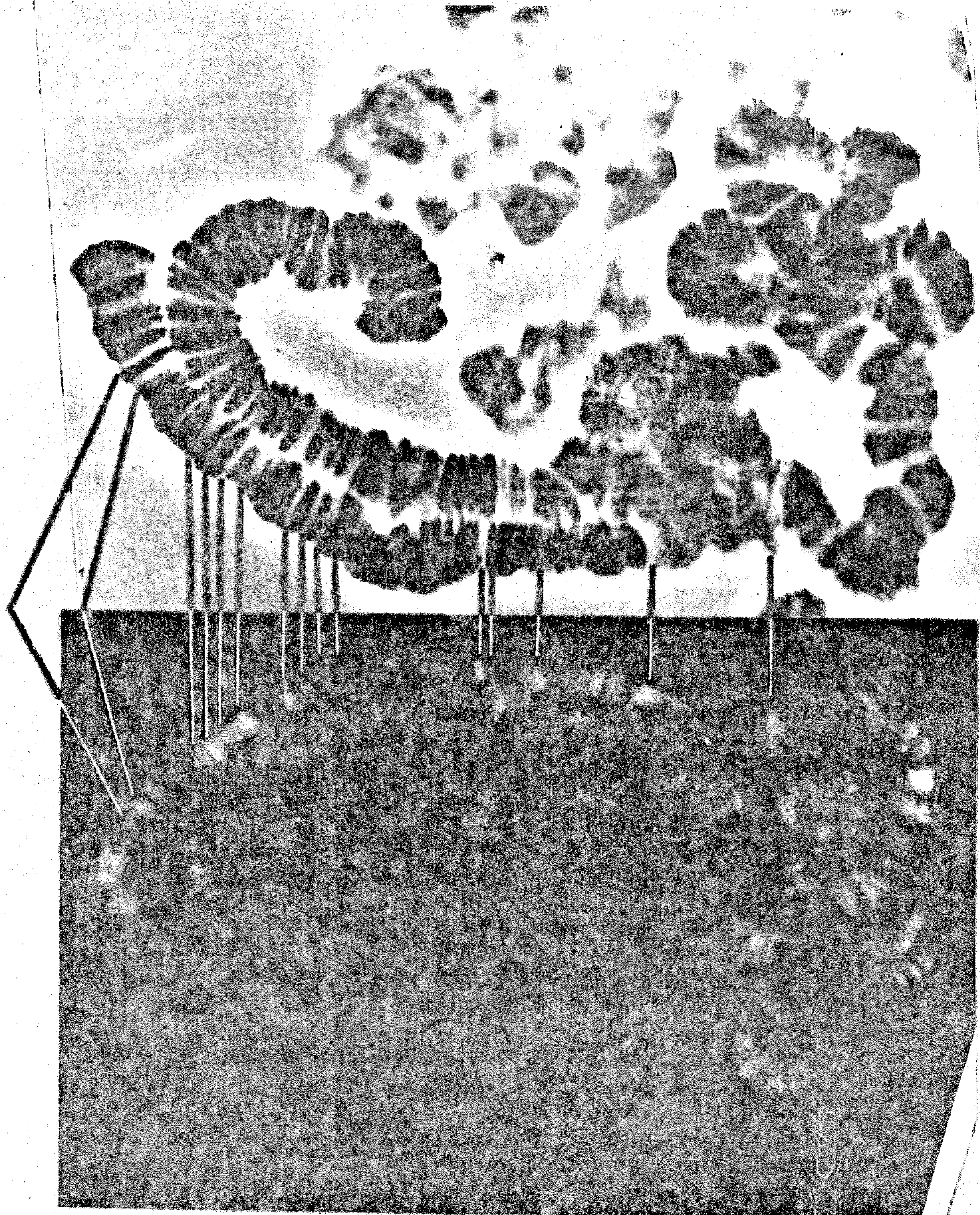

A

Figure 9. Comparison of indirect immunofluorescence staining with orcein staining of polytene chromosomes. Figure 8C has been reproduced here and juxtaposed with the phase contrast picture of the same chromosome arms stained with orcein. The image of the orcein stained pattern has been photographically reversed to facilitate comparison. The lines indicate the correspondence of fluorescent regions with interband regions of the chromosomes.

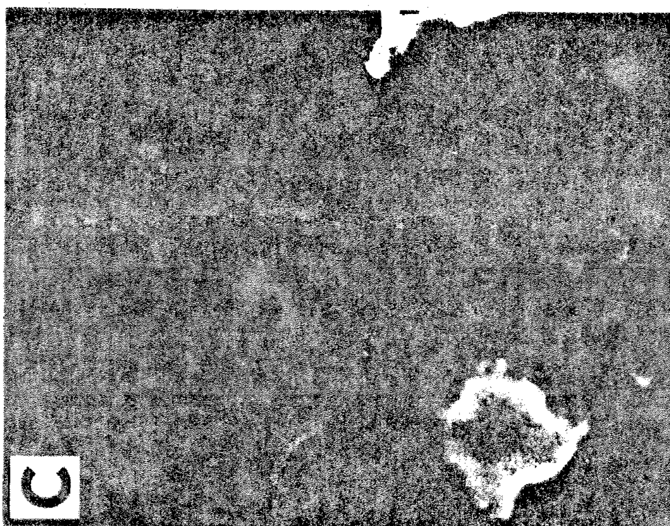


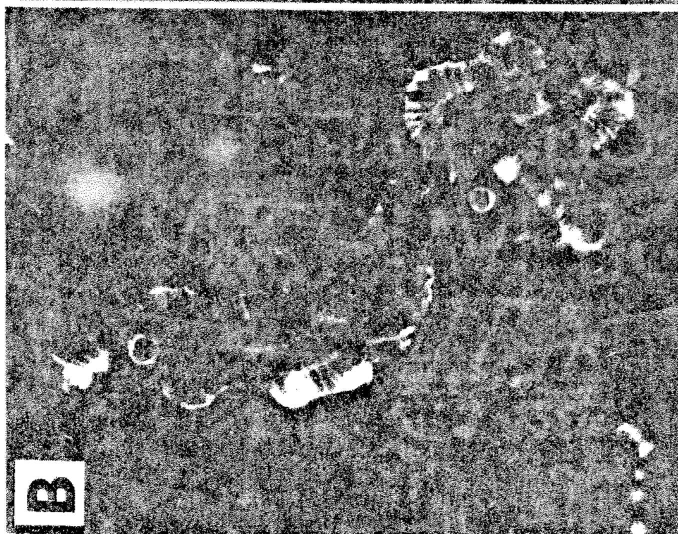
Figures 8B and 8C, whereas others on the same slide were stained only slightly (Figs. 10B, C). This variability within one slide was reproducible.

Since a slide of chromosome squashes prepared by the formaldehyde fixation technique was prepared from only one salivary gland, and the entire area of chromosome squashes was treated at one time with a given reagent, it seems unlikely that the variability is due to experimental artifact. Even two chromosome spreads very close to one another on the slide showed extremes of staining. In at least one such case, the second spread photographed was much more intensely stained than the first. Thus, failure to coat all of the spreads with the reagent solutions and inadvertent bleaching of the fluorescein can be ruled out as causes for the variability. For spreads in which the fluorescence staining was above control levels, a correspondence between fluorescent bands and interband regions of the chromosome was again observed.

Antiserum directed against the P protein at the same dilution as was used in the experiments represented in Figures 8 and 9, also gives an irregular banded pattern of fluorescence (Fig. 11). Here the intensity of staining is much greater than control levels, but not as great as in Figure 8. Since titrations of the antisera were not performed, one cannot say whether the difference in staining is due to different concentrations of the antigens on the chromosomes. It may be noted, however, that the cellular debris is stained more relative to the chromosomes when antiserum directed against the P protein is used (Figs. 10B, C), whereas the chromosomes are stained more relative to the debris when antiserum directed against the 84,000 dalton protein is used (Figs. 8B, C).

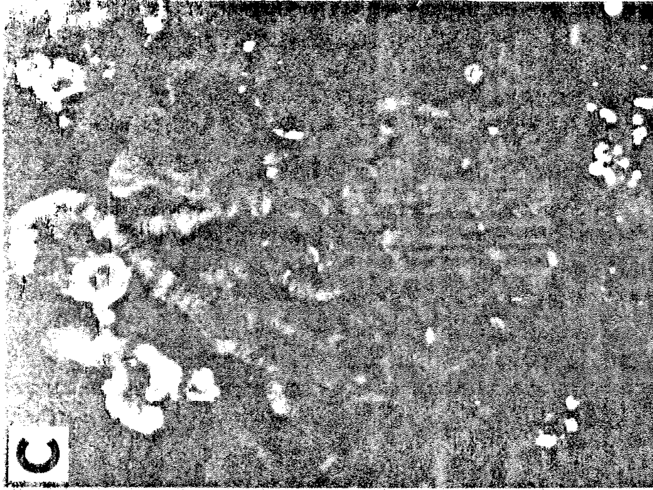
Figure 10. Indirect immunofluorescence staining of polytene chromosomes from heat shocked larvae using antiserum directed against the 84,000 dalton protein. Salivary gland chromosomes from larvae heat shocked at 37.5°C for 20 min were fixed by formaldehyde and stained using preimmune serum diluted 20 times (A), or antiserum diluted 20 times (B), (C). (B) and (C) are from the same slide.

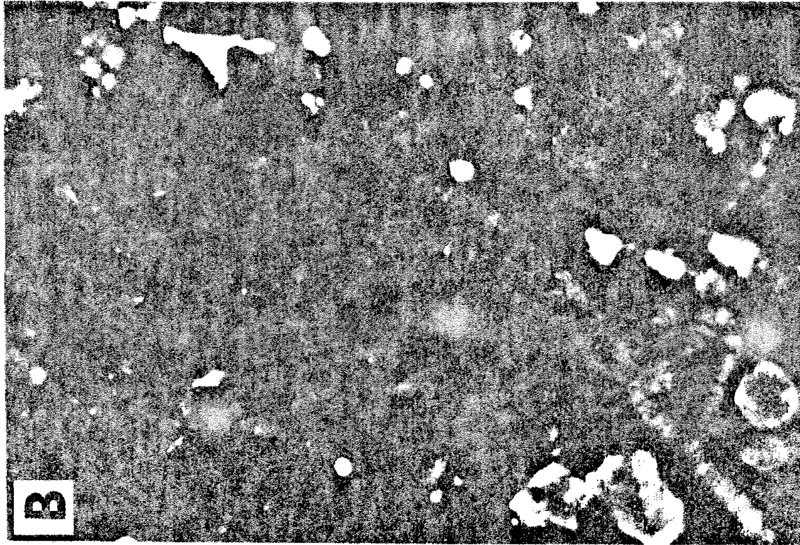

C

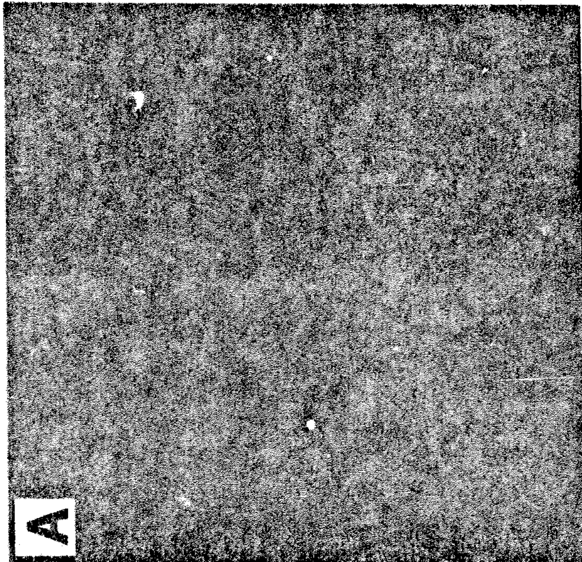

B

A

Figure 11. Indirect immunofluorescence staining of polytene chromosomes using antiserum directed against the P protein. Salivary gland chromosomes from larvae kept at 25°C were fixed by formaldehyde and stained using preimmune serum diluted 20 times (A), or anti-P serum diluted 20 times (B), (C). (B) and (C) are from the same slide.


C


B


A

Discussion

Hydroxylapatite is apparently an effective adsorbent for fractionating some of the labeled heat shock proteins. The 84,000, 70,000, and 68,000 dalton species are clearly enriched in some fractions.

The 70,000 dalton protein is purified in fractions 80-100 and this has been verified by two-dimensional electrophoresis of some of these fractions. The 34,000 dalton protein may be somewhat purified in fractions 50 to 58 but these have not been tested by two-dimensional gel electrophoresis.

It is difficult to say where the 27,000 and 26,000 dalton heat shock proteins elute, because of the complication introduced by the P protein. One would need to test for the presence of these proteins throughout the elution profile by two-dimensional gel electrophoresis, since no peak of radioactivity in the elution profile (Fig. 2) presents itself as a likely candidate.

The 23,000 and 22,000 dalton proteins do not seem to be purified very much in any of the hydroxylapatite fractions. If one wanted to pursue the purification of the lower molecular weight heat shock proteins, one might try different extraction buffers, since the one used in the experiments described here was tested only for adequate extraction of the 70,000 dalton protein from the tissues.

Mirault et al. (1) showed two-dimensional gel analysis of heat treated tissue culture cells and control cells. Cells kept at 25°C showed radioactive proteins migrating to positions corresponding to the 84,000 dalton and two 70,000 heat shock proteins (heat shocked tissue culture cells synthesize about five proteins of molecular weight 70,000 daltons), suggesting that these heat shock proteins are normally synthesized. Bishop (19) has reported that heat shock RNAs are also present, at much lower concentrations, in tissue culture cells kept at 25°C.

The work described here has shown that the 84,000 dalton heat shock protein can be synthesized normally in the animal. This normal synthesis of the protein may partly account for its making up 1% of the total protein in heat shocked flies 1 hr after heat treatment.

The indirect immunoprecipitation from NP-40 lysates showed that the 84,000 dalton protein is not bound to or associated with any other labeled protein, as, for example, a subunit of an enzyme. It is already known that none of the heat shock polypeptides is linked to another by disulfide bonds, since unreduced preparations of salivary glands run on SDS gels do not show any new labeled bands that could be combinations of heat shock polypeptides (data not shown). However the anti-84,000 dalton protein gamma globulins cause the precipitation of another polypeptide, of molecular weight 30,000 to 34,000, from SDS lysates of salivary glands from heat treated prepupae. No further experiments have been carried out to evaluate this phenomenon.

Since this 84,000 dalton protein is synthesized normally in at least some tissues, it is apparent that this is not a special protein present only in stressful situations but rather, it may be a common protein or enzyme. No information exists for how much the concentration of this protein is increased after exposure to heat. The stimulation of the synthesis of this protein may reflect an increased need for the protein or an adventitious disturbance in the regulation of its synthesis.

A clue to the molecular events which lead to the induced synthesis of this protein comes from data presented in a report on heat shock proteins in *D. hydei* (20). Normally an 84,000 dalton protein is not synthesized in *D. hydei* after exposure to heat or to other inducers of the heat shock puffing response (20, 21).

But Koninkx (20) found that in the presence of arsenite, the synthesis of the heat shock proteins, of molecular weights 70,000, 67,000, 38,000, 26,000, 25,000, and 21,000 and also of a protein of molecular weight greater than the 70,000 dalton protein is induced. Based on measurement of the mobility of this polypeptide on the gel relative to polypeptides of known molecular weight, this protein has a molecular weight of 84,000. This protein is almost certainly the correspondent of D. melanogster's 84,000 dalton heat shock protein, but no tests of immunological cross-reactivity have been carried out.

Since synthesis of this protein can only be induced by arsenite and not by vitamin B-6, by 2,4-dinitrophenol, or by exposure to anoxia, one might guess the physiological role of this protein, or, perhaps more easily, the identity of the inducer molecule(s) in the cell which is responsible for the stimulation of its synthesis.

It is likely that all of the chemical agents which can be used to induce the heat shock response (for review see 22) have side effects. Thus one must be cautious in attributing their action only to mechanisms involved in ATP formation or electron transport in mitochondria.

The results of indirect immunofluorescence staining experiments suggest that the 84,000 dalton protein is associated with the chromosomes, perhaps preferentially with respect to the cellular debris which is also fixed to the slides in these preparations. It is assumed that the antibodies bind only to the 84,000 dalton protein on the chromosomes. Indirect immunoprecipitation from NP-40 lysates of ^{35}S-methionine pulse-labeled salivary gland proteins results in specific selection of the 84,000 dalton protein from among other proteins by gamma globulins from antiserum directed against it. However antibody precipitation of unlabeled proteins

in less than amounts detectable by staining of gels would not have been observed. An indirect immunoprecipitation test using lysate proteins labeled in vitro with ^{125}I could be carried out, but might not add new light to the reactions on the chromosomes.

Whether this staining indicates a chromosomal role for this protein cannot be said at this point. The binding of the protein to the chromosomes may simply be a consequence of the relatively high concentration of the protein in the cell. The staining observed using the anti-P antiserum gives the same qualitative results. This protein is in even higher concentrations in the cell and one has no reason to believe it has a chromosomal function. On the other hand, Jamrich et al. (4) found by indirect immunofluorescence, that RNA polymerase was also located all over the chromosomes at the interband regions. This result suggested to the cited authors that the interbands are regions of transcription, albeit low level transcription, and moreover, that interband regions contain sites of initiation of transcription.

However, in heat shocked cells, the RNA polymerase is concentrated at the heat shock puff sites, and levels at interband regions are largely reduced (4). In the results presented here, no such rearrangement of the 84,000 dalton protein on the chromosomes was observed; instead, the same pattern of interband staining was observed, but at variable intensities. If this result is not due to experimental artifact, then one must conclude that the nuclei within one salivary gland are in different states with respect to the protein composition on the chromosomes.

The fact that formaldehyde fixation was necessary to detect the presence of the 84,000 dalton protein on the chromosomes may indicate that acid treatment

of the chromosomes may denature the protein in such a way as to make it no longer recognizable by antibodies directed against it. Alternatively, acid fixation may not have prevented changes in the morphology of the chromosomes, changes which could make the antigen inaccessible to the antibodies.

On the other hand, formaldehyde may have succeeded only in fixing cytoplasmic proteins to the chromosomes, to the most accessible sites, as suggested by the similar results obtained with antisera to the 84,000 dalton and to the P proteins.

The experiments using the indirect immunofluorescence technique suggest other experiments using antibodies to the 84,000 dalton protein and immunological stains such as fluorescent goat anti-rabbit immunoglobulin antibodies or peroxidase-anti-peroxidase (23) to locate the antigen in fixed cells or thin sections of cells. Association with particular structures may give information regarding the physiological role of this protein.

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CHAPTER 5

HEAT SHOCK RESPONSE IN HeLa CELLS

Introduction

In organisms other than Drosophila changes in the synthesis of macromolecules after heat or anoxia treatment have been reported. For example, Tetrahymena cells, normally grown at 28°C, stop synthesizing the precursor to ribosomal RNA when exposed to 34°C (1). Zea mays cells, after long-term exposure to anaerobic conditions, synthesize several new proteins, one of which is a subunit of alcohol dehydrogenase. At the same time, the labeling of many normally synthesized proteins is reduced (2).

McCormick and Penman in 1969 (3) reported that the polysomes of HeLa cells, normally grown at 37°C, disaggregated shortly after exposure of the cells to 42°C. After longer exposure, polysomes reformed, but in lower amount than normally present at 37°C. The same phenomenon was reported by McKenzie et al. (4) for D. melanogaster tissue culture cells exposed to elevated temperature. McCormick and Penman (3) analyzed the electrophoretic profile of proteins synthesized by HeLa cells at 42°C to investigate whether the polypeptides synthesized on polysomes at this temperature were abortive translation products. They found that the average size of the polypeptides synthesized at 42°C was very similar to that of polypeptides synthesized at 37°C. They found some differences in the electrophoretic profiles, but their gels gave poor resolution of the proteins and were not run with any molecular weight standards. It was therefore considered interesting to reexamine, under more appropriate conditions, the size distribution of the HeLa cell proteins synthesized at elevated temperatures. These were run

on SDS slab gels to see whether any drastic change in the normal protein synthesis pattern occurs in HeLa cells at higher temperatures; furthermore, the pattern of these proteins was compared with a typical D. melanogaster heat shock pattern to see whether any heat induced proteins in HeLa cells correspond in mobility to D. melanogaster heat shock proteins. It had been determined previously that survival of HeLa cells kept at 42°C for 20 min was decreased by 10%; after 50 additional min at that temperature the survival was reduced to 65%. At 44°, survival was decreased by 25% after 20 min, and by 85% after 70 min (G. Attardi, personal communication). Both of these temperatures were used in the protein synthesis experiments.

Materials and Methods

Tissue culture cells

Exponentially growing HeLa cells (strain S_3) were used for these experiments. The cells were concentrated to 5 x 10^5 cells/ml in methionine-free Dulbecco modified Eagle's phosphate medium supplemented with 5% dialyzed calf serum.

Heat shock and labeling

One ml aliquots of cells were distributed into 5 ml volumetric flasks and kept at 37°C (1 flask) or immersed in water baths set to 42°C (2 flasks) or 44°C (2 flasks). After 20 min at each temperature, 25 µl containing 50 µCi of ^{35}S-methinine (240 Ci/mmole, New England Nuclear) in 0.1% β-mercaptoethanol was added to each flask. One of the pair of flasks at 42°C was kept at 42°C for another 50 min; the other was transferred to 37°C and labeled at 37°C for 50 min. The flasks at 44°C were treated in the same way: one was kept at 44°C for an

additional 50 min to label, and the other was transferred to 37°C for 50 min labeling. Labeling was stopped by dilution into ice cold NKM solution (0.13 M NaCl, 0.005 KCl, 0.001 M $MgCl_2$). The cells were washed in NKM and precipitated with 10% TCA. The pellets were extracted successively with 95% ethanol and a 1:1 mixture of chloroform and methanol, and dissolved in the same sample buffer as used for salivary glands in Chapter 1a; portions were then run on SDS gels as described in Chapter 1a. Approximately the same number of counts was run, in the same volume, in each lane of the gel.

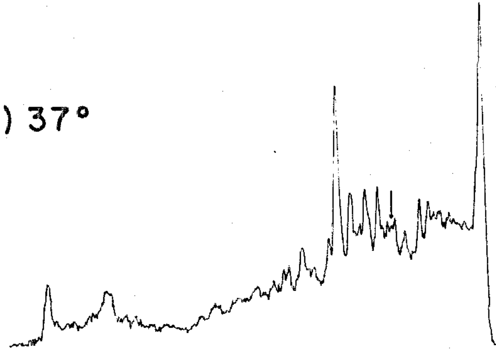
Results and Discussion

From Figure 1 it can be seen that all of the HeLa cell samples exposed to temperatures higher than 37°C show a change in the electrophoretic profile of pulse-labeled proteins, particularly near the position of migration of the 70,000 dalton D. melanogaster heat shock protein. The cells kept at 37°C (Fig. 1a) synthesize several major proteins which migrate faster than the 70,000 dalton protein, one of which gives a particularly high peak on the densitometer scan, whereas cells exposed to 42°C for 20 min, 42°C for 70 min, or 44°C for 20 min (Figs. 1b, c, d), synthesize a protein just slightly slower moving than the 70,000 dalton protein, in addition to the major proteins synthesized by cells at 37°C. The profile of proteins synthesized in cells kept at 44°C for 70 min is shown in Figure 1e. The protein synthesis pattern is more altered in this sample than in any of the other heat shocked samples. The major peak has been quite reduced in its height relative to the rest of the profile and the sharp peak just to the right of the arrow in panels b, c, and d is reduced or missing.

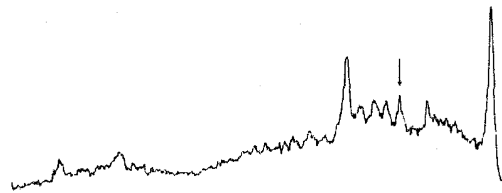
Figure 1. Densitometric scans of autoradiogram of HeLa cell proteins labeled with ^{35}S-methionine during or after exposure to high temperature. The panels are labeled as follows: (a) cells kept at 37°C and pulse-labeled for 50 min; (b) cells kept at 42°C for 20 min, then immediately labeled for 50 min at 37°C; (c) cells kept at 42°C for 20 min, then labeled at 42°C for an additional 50 min; (d) cells kept at 44°C for 20 min, then labeled for 50 min at 37°C; (e) cells kept at 44°C for 20 min, then labeled at 44°C for an additional 50 min.

The direction of migration through the gel is from right to left. The arrow indicates the position of migration of the 70,000 dalton heat shock protein in a salivary gland sample run on the same gel. Interruptions in the scans are due to cracks in the gel.

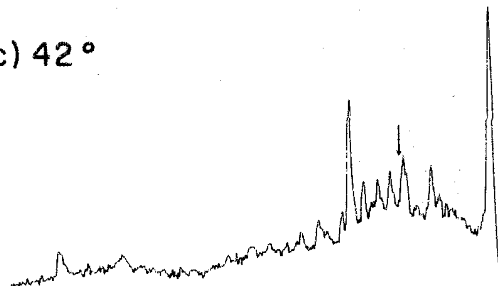
a) 37°



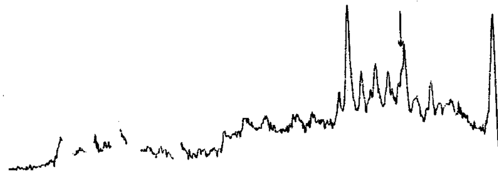
b) 42°



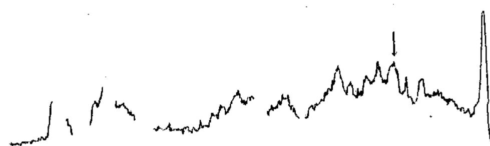
c) 42°



d) 44°



e) 44°



Thus HeLa cells are affected in the pattern of protein synthesis after heat shock, and may even have a "heat shock protein" of molecular weight slightly greater than 70,000 daltons. However, the abrupt decrease in normal protein synthesis after heat shock normally seen in *D. melanogaster* is not evident here. After 70 min at 44°C there is a marked alteration in the pattern of protein synthesis in HeLa cells; after this treatment, however, only 15% of the cells survive. Heat shock in HeLa cells, then, does not present itself as a simple model system for studying induction and suppression of gene expression, since neither occurs, after this treatment, in the extreme form observed in *D. melanogaster*.

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Abbreviations

BSA	bovine serum albumin
DTT	dithiothreitol
cpm	counts per minute
EDTA	ethylenediamine tetraacetic acid
-K protein	-thousand dalton protein
MOPS	morpholinopropane sulfonic acid
MWt	molecular weight
NP-40	nonidet P-40
OD	optical density
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane