DEVELOPMENT AND PROTEIN SYNTHESIS IN *DROSOPHILA*

Thesis by

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To my sister Nadya, my daughter Karen, and Dan.

"We dissect flies," said the philosopher, "we measure lines, we assemble numbers, we agree about two or three points that we understand, and we argue about two or three thousand that we do not understand."

Francois Marie Arouet de Voltaire

"Micromegas"

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It is difficult to start, for there are so many...it is hard to say who was the most important...

The impact my permanent residence visa had on all my activities is impossible to describe, so essential was it to me to have the security of not being suddenly deported within 24 hours. Here is the list of people (in alphabetical order) who worked for it and demonstrated to me that difficult situations can be solved and impossible ones can also be solved with a little extra time and extra persistence: Dr. James F. Bonner, Dr. Leroy E. Hood, Dr. Robert A. Huttenback, Mrs. Ellen Kennedy and Dr. Oscar Mandel. I thank them all.

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ABSTRACT

CHAPTER 1

At various times during metamorphosis, brain tissue of D. melanogaster has been pulse-labeled in situ with ^{35}S-Met. The composition of the newly synthesized stage-specific proteins and of the total bulk of Coomassie stained polypeptides present at the same time in the tissue, or the parts of it, were analyzed on SDS-polyacrylamide gels.

The striking similarity and constancy of the obtained patterns, and the finding that most of the observed accumulating polypeptides are glycosalated, lead to the conclusion that the predominant change in the larval Drosophila brain is dendritic and/or axonal reorganization.

CHAPTER II

Pupae of Drosophila melanogaster were heat shocked under conditions required to induce phenocopies in more than 90% of the flies that subsequently emerge. The effects of these treatments on protein synthesis in two tissues (thoracic epithelium and brain) were followed for several hours after the heat treatments. Results from pulse labeling and protein separations on SDS acrylamide gels showed a virtually complete cessation of protein synthesis immediately after the shock followed by a non-coordinate resumption of the starting pattern. Similar experiments following double heat shocks demonstrated a more rapid resumption of synthesis of heat shock proteins after two successive heat treatments than after a single one.

CHAPTER III

We describe variants of three heat shock proteins of Drosophila melanogaster and the use of these to map the chromosome regions which contain the coding

sequences for these proteins. All three map to a region on chromosome 3L which includes only one heat shock puff, that designated as 67B. The results imply that the genes which code for at least three heat shock proteins are included within the 67B region.

CHAPTER IV

Mild heat treatments applied to whole animals or cell cultures of Drosophila melanogaster prior to lethal heat shocks result in both survival and protection against phenocopy induction. From an examination of these heat shock effects on transcriptional and translational activities in tissues and cells it appears that the protective action of pretreatment is due to sequestering of mRNAs in a masked form as RNPs. Heat shock proteins are evidently involved in the masking process either directly or indirectly.

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CHAPTER I

Development and protein synthesis in *Drosophila* brain during metamorphosis

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INTRODUCTION

A particular advantage of studying an organism such as Drosophila lies in the fact that metamorphosis provides distinct stages of differentiation and development that can be analyzed on the molecular level with relative ease. The entire transformation lasts only four days. Because of its relative simplicity and ease of handling in the laboratory and extensive genetical data available including the complete library of the genome, Drosophila makes an attractive candidate for an intensive study of the molecular mechanisms controlling development in a eukaryote. The possibility of biochemical analysis of a nervous tissue with all of the above mentioned characteristics of the organism inspired the authors of this work.

Drosophila brain is one of the few tissues that survives metamorphosis and unlike most other larval organs of Diptera or Lepidoptera, the larval nervous system does not undergo extensive hystolysis during metamorphosis (1). To provide some of the biochemical background for studies of development of the Drosophila brain during this period, a study of the changes in protein synthesis during metamorphosis was undertaken, using pulse-labeling of the tissue in situ and analysis of protein patterns on SDS-polyacrylamide gels.

MATERIALS AND METHODS

Abbreviations Used

Con A, concanavalin A; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; MOPS, morpholinopropane sulfonic acid; MWt, molecular weight; RCA-60, Ricinus communis agglutinin-60; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; WGA, wheat germ agglutinin.

Fly Stock and Culture

Wild type stocks of Canton S Drosophila melanogaster were raised in mass culture at 25°C as described by Mitchell and Mitchell (2). Eggs were collected for 2 hr. During the final 12 hr of the 3rd instar stage, the larvae stopped eating and crawled out of the food to the top of the culture box, from which they were collected for dissection. White prepupae (time-0) were picked out as soon as sclerotization occurred and dissected immediately.

Animals were synchronized at 5 hr after puparium formation by floatation (2) in tap water at 21-22°C. In quite a few preliminary experiments, the selection of synchronized animals was done with 30-45 min intervals. In all experiments presented here, the interval between floats was 1 hr. Subsequently, the synchrony of animals was monitored as follows: for 12 hr pupae, only animals with everted heads were used; for 24 hr pupae, the shape of the brain and the thoracic ganglion was the guide; 36 hr pupae were chosen on the basis of the morphology of the brain and completeness of eye formation; 48, 60 and 72 hr pupae were characterized by eye pigmentation; 84 hr pupae were selected according to pigmentation of the thorax, wings and abdomen. The fly was dissected as soon as it emerged from the pupal case. All animals used in the experiments were synchronized to at least $\pm$ 1 hr of the indicated times.

Dissection and Labeling

While dissecting, brains were moved from one drop to another of MOPS buffered medium (3) until the tissue was completely cleaned of attached fat bodies and, in the case of larval and pupal brains, of ring glands and eye discs as well. In later stages of development, eyes were removed prior to incubation. For experiments in which labeling was involved, it was crucial to complete dissections within 15 min, since the label incorporation remains essentially constant during

the first 30 min, but decreases slightly (~20%) during the next 30 min and becomes negligible by two hours after dissection. In all experiments presented here, tissue was placed in the incubation medium not later than 15-20 min after the beginning of dissection.

The tissue was incubated for 25 min at 23-25°C in 2.5-3 µl of MOPS buffered medium containing about 20 µCi of ^{35}S-methionine of specific activity 570 Ci/mMol (New England Nuclear). After incubation, the tissue was washed three times with MOPS buffered medium and transferred to a chip of soliconized coverslip. The chip was placed in a small test tube, to which 30 µl of SDS sample buffer was added. Sample buffer was prepared according to Fairbanks et al. (4) with the following modifications: the buffer contained 10 mM EDTA, was at pH 7.75, and the DTT was freshly added to the sample buffer for each experiment. Within 1-2 hr of standing at room temperature, the tissue was completely dissolved. Subsequently, samples were kept frozen at -80°C for not longer than 4-5 days. Immediately prior to electrophoresis, the samples were heated at 37°C for 20 min. Samples prepared in this manner, or by 10% TCA precipitation followed by washing with 95% ETOH (5), showed the same electrophoretic properties. Electrophoretic patterns remained unchanged when 3 to 6 brains were used for each sample.

Separation of the eye brain from the remainder of the hemisphere is possible even in the larval and prepupal stages since the demarcation between the two is clearly visible. To do so, the eye disc is gently pulled with forceps while holding the adjacent portion of the hemisphere. Upon stretching during this operation, the brain resembles the more clearly divided form of later developmental stages which are shown in Fig. 1 (C and D).

In those experiments in which parts of the brain were run separately on the gel, the brains were incubated intact and washed as usual, then the parts were

separated. The entire operation added 5-7 min to the procedure for four brains. Cold brains were dissected and their parts were combined with the corresponding parts of labeled brains. The electrophoretic patterns of male and female brains for all stages were alike.

Gel Electrophoresis

Electrophoresis was carried out on 1 mm-thick 24 cm-wide slab gels with a 1 cm-base using a constant current of 12 mA. The SDS-containing buffers of Laemmli (6) were used. The base was made with 20% acrylamide and 0.2% bis-acrylamide. The exponential gradient of 10-20% in acrylamide (Scientific) and of 0.2-0.27% bis-acrylamide (Scientific) for the separating gel was made according to Van Blerkom and Manes (7). The stacking gel was 6% in acrylamide and 0.08% in bis-acrylamide. Staining and destaining were carried out as described by Fairbanks et al. (4). Gels were photographed, then dried and exposed for autoradiography on Kodak SB-5 No Screen X-ray film. In order to present the complete pattern seen on the autoradiogram in Fig. 2B, longer exposures were used in the later stages of development. Figure 2B, where the intensity of the bands has been tabulated, is based on an autoradiogram of 23 hr 40 min exposure.

Molecular Weight Determination and Band Identification

Heat-induced proteins (5) from larval salivary glands and brains were used as standards on each gel. The molecular weights of the heat shock proteins were taken from Mirault et al. (8) and the least squares-fitted line (correlation coefficient = 0.9993) of log molecular weights versus mobility was used to determine the molecular weights of the other proteins on the autoradiogram. Since the autoradiography was done on the dried gel while the Coomassie-stained pattern was obtained from the wet gel, a separate least squares line

(cc = 0.9999) was constructed. For this, some heat shock proteins and prominent bands visible on the autoradiogram were identified with the protein bands visible on the dry gel by direct superposition, and these bands were used as standards in making the least squares-fitted line for Fig. 2A. When the molecular weights were determined for all polypeptides on the autoradiogram and on the photograph of the wet gel, each band from the autoradiogram was identified with its counterpart on the dry gel using a compass for accurate distance measurement. For all the bands present on both, the molecular weight average of the two was assigned. For those cases in which a band appeared in only one of the two photographs (Coomassie-stained and autoradiogram), the MWt was estimated by an interpolative procedure as follows: the differences in MWts between the two photographs for the two adjacent bands were measured and the position of the absent band was estimated by linear interpolation. The MWt was then assigned as an average of the measured and estimated values.

Lectin Diffusion

Bovine γ-globulin was from Nutritional Biochemical Corp., avidin ovalbumin, ribonuclease, ovomucoid, N-acetyl-D-glucosamine, and β-lactose are from Sigma, alpha-methyl-D-mannopyranoside from Calbiochem.

Gel electrophoresis was carried out as described above except 18 mA of constant current was used, and an aliquot containing seven brains of 5.5-7 hr prepupae or 89-91 hr pupae was applied in each slot. Glycoproteins with known carbohydrate composition [bovine γ-globulin (9-11), ovalbumin (12), ribonuclease (13), ovomucoid (14, 15)] or known for the presence of lectin receptor(s) [avidin (16)] were used as standards in concentrations of 10 μg of each per slot.

The position of glycoproteins in SDS polyacrylamide gels was determined by staining with several fluorescent lectins as described by West et al. (16). The

carbohydrate binding specificity of these lectins was checked by carrying out parallel incubations in the presence of hapten sugar inhibitors. The monosaccharides N-acetyl-D-glucosamine, alpha-methyl-D-mannopyranoside and β-lactose were used in conjunction with the lectins wheat germ agglutinin, concanavalin A, and Ricinus communis agglutinin-60, respectively. Fluorescence photography was modified so that the light source was a slide projector (Ektagraphic, Kodak). Excitation wavelengths were selected using an interference filter with a 20 nm bandwidth centered at 480 nm and emission wavelengths were selected with a second interference filter with a 20 nm bandwidth centered at 520 nm (Omega Optical, Brattleboro, Vt.). Light of wavelength 500 nm passing through both filters simultaneously was 10^{-7} of the intensity passed by either filter alone at 480 nm and 520 nm, respectively.

Electron Micrograph Scan

Brains were dissected in MOPS buffered medium (2) and fixed and dehydrated according to Karnovsky (17). After critical point drying from CO_2, brains were mounted on silver paste (Pelco Colloidal 16032), coated with gold on a rotary platform, and viewed on an ETEC scanning electron microscope.

RESULTS

Morphology

At the third instar and early prepual stages, the structure of the Drosophila brain is relatively simple, consisting of two brain hemispheres and a ventral ganglion with attached nerve fibers (Fig. 1A and C). At about 10 hr after puparium formation, the portion of ventral ganglion adjacent to the brain hemisphere begins to constrict and one first perceives the separation of each

Figure 1. Drosophila brain at various stages of development.

A,B. Late 3rd instar larvae or white prepupae. (1) - ring gland, (2) - brain hemisphere, (3) - ventral ganglion, (4) - nerve fibers.

C. Prepupal brain (10 hr). (5) - connection to the eye disc, eyestalk, (6) - constriction of ventral ganglion, which leads to formation of cephalothoracic chord.

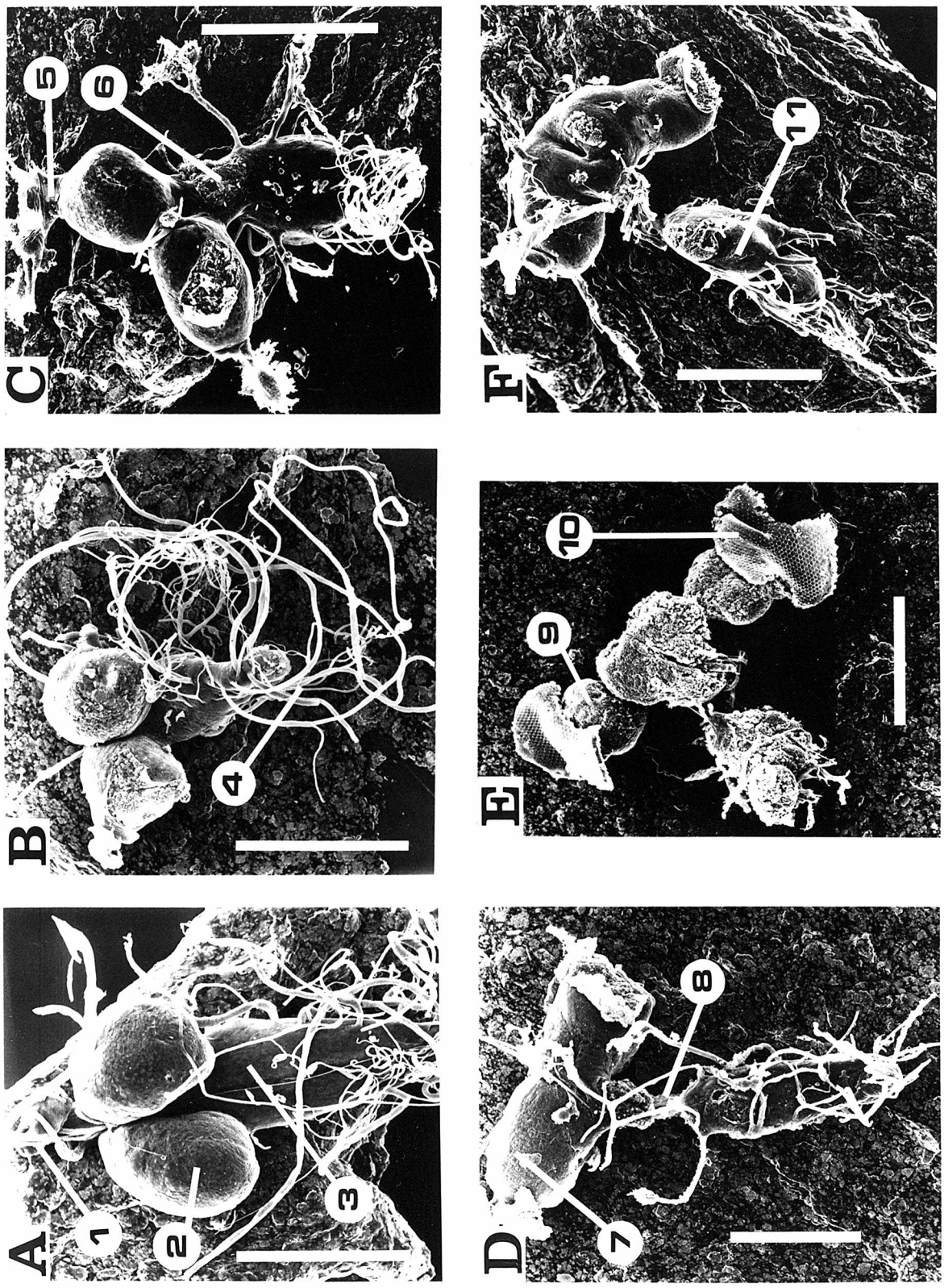
D. Pupal brain (13-14 hr). (7) - pronounced appearance of the eye ganglia, (8) - cephalothoracic chord.

E. Pupal brain (36-38 hr). (9) - well formed eye ganglia separated from brain hemisphere, (10) - compound eye.

F. Pupal brain (81 hr). Morphologically identical with the brain of an adult fly. (11) - thoracic ganglion. Note that eye ganglia and brain hemispheres are again fused.

The compound eye is removed in this picture.

0 hr - time of puparium formation. White bar corresponds with 300 microns.


A
1
2
3
B
4
C
5
6
D
7
8
E
9
10
F
11

hemisphere into two incipient lobes (Fig. 1C). By the 13th hour after puparium formation, the constriction of the ventral ganglion to form the cephalothoracic chord is virtually complete, and the hemispheres have become elongated; by the 24th hour after puparium formation, the optic lobe has emerged as a well-separated spherical structure. This loose attachment of the eye ganglia persists through the 36th hour (Fig. 1E) by which time the compound eye has completely developed. Soon the brain hemispheres and the eye ganglia become fused again, but remain as distinct structures in the adult brain. This fusion is a gradual process which is completed by about 80 hr after puparium formation and is accompanied by a progressive differentiation of the thoracic ganglion into three lateral sections (Fig. 1F).

Protein Patterns of Drosophila Brain during Metamorphosis seen on SDS Polyacrylamide Gels

Brain tissue at various morphological stages has been analyzed for protein composition by polyacrylamide SDS gel electrophoresis. For a comparison of band patterns, brain samples were taken at 12 hr intervals. This allowed inclusion on the same gel of samples spanning 100-110 hr of Drosophila life, starting with late 3rd instar larvae and continuing up to adult (newly emerged) flies.

The 20 min pulse-labeling in situ provides information about newly synthesized polypeptides at the specified time of development (Fig. 2B) whereas Coomassie staining reveals the total bulk of polypeptides present at the same time in the tissue (Fig. 2A).

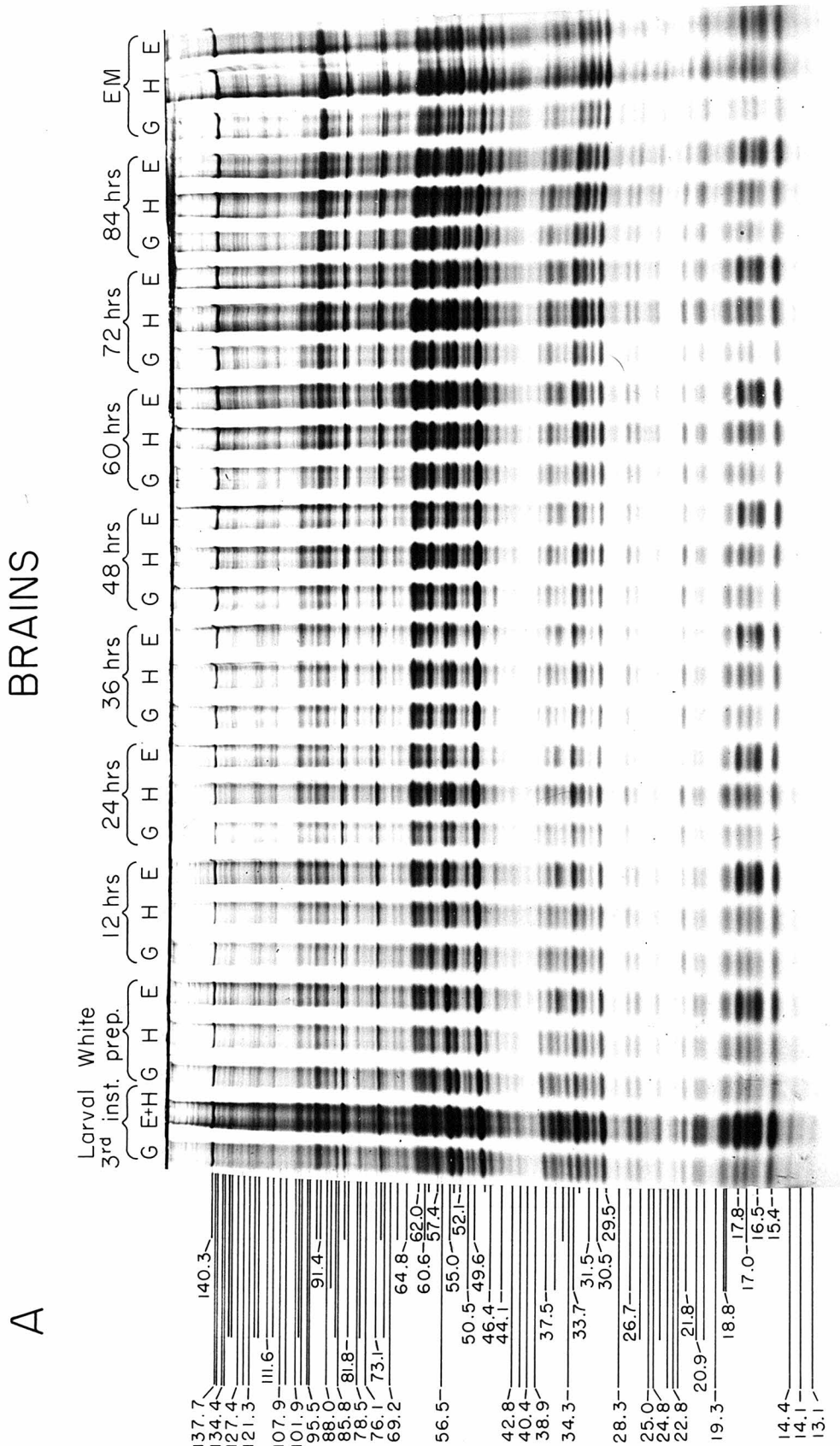
From a casual inspection of Fig. 2A and B, one can notice a striking similarity in band patterns of Coomassie-stained and pulse-labeled polypeptides. A very detailed and careful analysis shows that for only five (corresponding to MWts 37,500, 34,300, 25,000, 24,800, and 22,800) out of approximately 90 resolved

Figure 2. Stage-specific protein patterns for parts of Drosophila melanogaster brain during metamorphosis.

A. Coomassie staining of 10-20% polyacrylamide exponential gradient SDS gel.

B. Autoradiogram of the same gel (^{35}S-met-label).

Parts of brain are designated as: G - ventral or thoracic ganglion, H - hemisphere, E - eye lobe, without eye. Ages of the pupae are given as hours after puparium formation. EM designates just emerged flies taken for dissection. Numbers on the left of the figure represent MWt x 10^{-3} estimated as described in Materials and Methods. Bands were divided into five arbitrary groups of different intensities, the molecular weights of which have been separated into columns above, with the intensity increasing from left to right.


A
BRAINS
Larval White
3rd inst. prep.
G E+H G H E
12 hrs
G H E
24 hrs
G H E
36 hrs
G H E
48 hrs
G H E
60 hrs
G H E
72 hrs
G H E
84 hrs
G H E
EM
G H E
140.3
137.7
134.4
127.4
121.3
111.6
107.9
101.9
95.5
91.4
88.0
85.8
81.8
78.5
76.1
73.1
69.2
64.8
62.0
60.6
57.4
56.5
55.0
52.1
50.5
49.6
46.4
44.1
42.8
40.4
38.9
37.5
34.3
33.7
31.5
30.5
29.5
28.3
26.7
25.0
24.8
22.8
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17.0
16.5
15.4
14.4
14.1
13.1

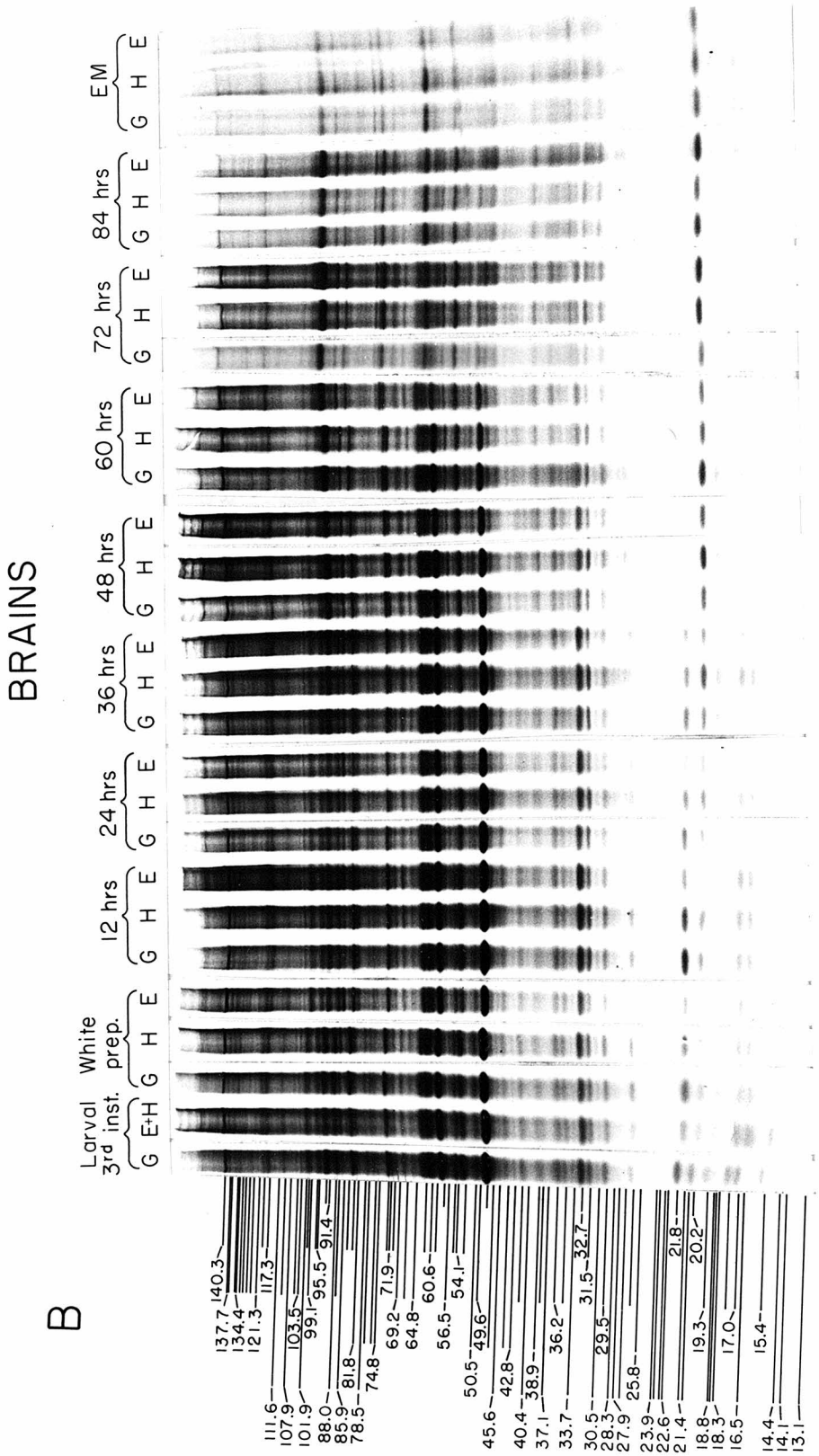

B
BRAINS
Larval 3rd inst.
White prep.
12 hrs
24 hrs
36 hrs
48 hrs
60 hrs
72 hrs
84 hrs
EM
G E+H
G H E
137.7
140.3
134.4
121.3
117.3
111.6
107.9
103.5
101.9
99.1
95.5
91.4
88.0
85.9
81.8
78.5
74.8
69.2
71.9
64.8
60.6
56.5
54.1
50.5
49.6
45.6
42.8
40.4
38.9
37.1
36.2
33.7
31.5
32.7
30.5
29.5
28.3
27.9
25.8
23.9
22.6
21.8
21.4
18.8
19.3
20.2
18.3
17.0
16.5
15.4
14.4
14.1
13.1

Table 1. Molecular weights x 10^{-3} of polypeptides appearing on Fig. 2a and b

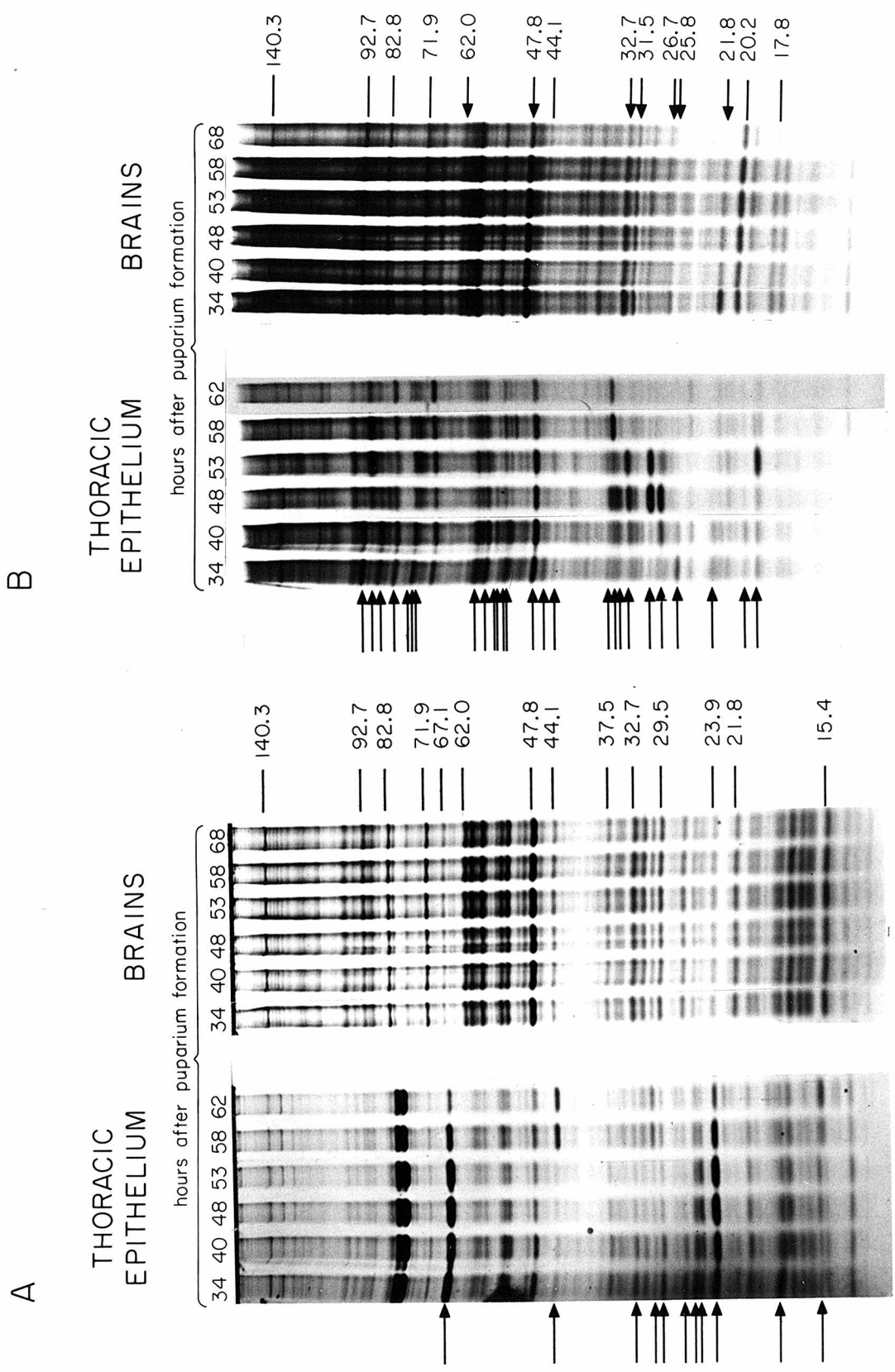
Band number	Bands on Coomassie stained gel	Bands on autoradiogram	Band number	Bands on Coomassie stained gel	Bands on autoradiogram	Band number	Bands on Coomassie stained gel	Bands on autoradiogram	Band number	Bands on Coomassie stained gel	Bands on autoradiogram	Band number	Bands on Coomassie stained gel	Bands on autoradiogram
1.	140.3	140.3	20.	92.7	92.7	39.	59.7	59.7	58.	36.2	36.2	77.	22.6	22.6
2.	137.7*	137.7*	21.	91.4	91.4	40.	57.4	57.4	59.	34.8	34.8	78.	21.8	21.8
3.	134.4*	134.4*	22.	88.0	88.0	41.	56.5	56.5	60.	34.3	-	79.	-	21.4
4.	131.1	131.1	23.	87.8	87.8	42.	55.0	55.0	61.	33.7	33.7	80.	20.9	20.9
5.	130.4*	130.4*	24.	85.8	85.8	43.	54.1	54.1	62.	32.7	32.7	81.	20.2	20.2
6.	127.4	127.4	25.	82.8	82.8	44.	52.1	52.1	63.	31.5	31.5	82.	19.3	19.3
7.	124.9	124.9	26.	81.8	81.8	45.	50.5	50.5	64.	30.5	30.5	83.	18.8*	18.8*
8.	121.3	121.3	27.	78.5	78.5	46.	49.6	49.6	65.	29.5	29.5	84.	-	18.3
9.	119.4	119.4	28.	77.9	77.9	47.	47.8	47.8	66.	28.3	28.3	85.	17.8	17.8
10.	117.3	117.3	29.	76.1	76.1	48.	46.4	46.4	67.	-	27.9	86.	17.0	17.0
11.	111.6	111.6	30.	-	74.8	49.	-	45.6	68.	-	27.4	87.	16.5	16.5
12.	109.9	109.9	31.	73.1	73.1	50.	44.1	44.1	69.	26.7	26.7	88.	15.4	15.4
13.	107.9	107.9	32.	71.9	71.9	51.	42.8	42.8	70.	25.8	25.8	89.	14.4	14.4
14.	105.3	105.3	33.	70.7	70.7	52.	41.3	41.3	71.	25.0	-	90.	14.1	14.1
15.	-	103.5	34.	69.2	69.2	53.	40.4	40.4	72.	24.8	-	91.	13.1	13.1
16.	101.9	101.9	35.	67.1	67.1	54.	38.9	38.9	73.	23.9	23.9			
17.	101.1	101.1	36.	64.8	64.8	55.	-	38.1	74.	-	23.7			
18.	99.1	99.1	37.	62.0	62.0	56.	37.5	-	75.	23.2	23.2			
19.	95.5*	95.5*	38.	60.6	60.6	57.	-	37.1	76.	22.8	-			

*Doublet

Figure 3. Comparison of the electrophoretic patterns of brain and thoracic epithelium proteins of D. melanogaster within the same range of developmental stages.

A. Coomassie stained pattern.

B. Autoradiogram of ^{35}S-methionine pulse-labeled proteins from a 10-20% exponential gradient polyacrylamide SDS gel. Each sample consisted of tissue dissected from three specimens. Arrows indicate the bands undergoing changes in intensity as development proceeds. Molecular weights are calculated as described in Materials and Methods.


A
THORACIC EPITHELIUM
BRAINS
hours after puparium formation
34 40 48 53 58 62 68
140.3
92.7
82.8
71.9
67.1
62.0
47.8
44.1
37.5
32.7
29.5
23.9
21.8
15.4
B
THORACIC EPITHELIUM
BRAINS
hours after puparium formation
34 40 48 53 58 62 68
140.3
92.7
82.8
71.9
62.0
47.8
44.1
32.7
31.5
26.7
25.8
21.8
20.2
17.8

bands (Coomassie staining), there exist no counterparts among the ^{35}S-Met-labeled polypeptides. Similarly, only 10 out of 90 bands on the autoradiogram (103,500, 74,800, 45,600, 38,100, 37,100, 27,900, 27,400, 23,700, 21,400, and 18,300) could not be detected at any investigated stage among Coomassie-stained polypeptides. All the bands seen in Fig. 2A and B, starting from MWt 140,300, are compiled in Table 1.

The main and most easily observed feature of both plates A and B of Fig. 2 is an almost complete constancy in band patterns. A very gradual increase or decrease in intensity of the bands throughout the time of metamorphosis is observed rather than dramatic changes as noticed in other tissues [salivary glands (5), malpighian tubes (18), flight muscles (19), thoracic epithelium (20, 21), see Fig. 3)]. In the experiments performed with shorter intervals (2 hr apart between the samples), the pattern for Coomassie-stained and ^{35}S-Met-labeled polypeptides remained the same, i.e., there was no evidence of any transiently existing and/or synthesized polypeptides in Drosophila brain during metamorphosis within the limitation of the technique (data not shown).

Since 94% of the total number of the resolved polypeptide species are seen as newly synthesized stage-specific polypeptides (Fig. 2B) and are also present as cold polypeptides at the same time in the tissue, and 93% have the same or nearly the same relative intensity, including the most pronounced ones (82,800, 57,400, 62,000, 59,700, 54,100, 52,100, 47,800, 32,700), it was of interest to compare the course of synthesis throughout metamorphosis of each particular component with the total Coomassie-staining material migrating to the same position on the gel. One band does not necessarily represent one type of polypeptide, but this ambiguity does not affect the analysis to follow. Making this comparison, one has to keep in mind that the intensity of a band seen on the autoradiogram

represents the degree of ^{35}S-Met incorporation into a particular polypeptide species and the rate of synthesis of this polypeptide at the given time of development. The amount of material present in even the most intense bands on the autoradiogram contributes a small or negligible proportion to the intensity of the band seen in the Coomassie-stained pattern at the same position. On cursory inspection of Fig. 2A and B, the most obvious difference between the two patterns is the displacement in intensity of the bands during the course of development: the bands on Fig. 2A gain in intensity towards the end of metamorphosis (starting from 48 hr old pupae), whereas the reverse is observed on the autoradiogram. At the same time in development, the intensity of the bands seen on the autoradiogram starts to decrease rapidly and continues to do so up to the end of metamorphosis. Bands 20,200, 58,700 and 73,100 (Fig. 2B) are exceptions, but they do not change the general picture. This decrease and/or disappearance of certain components seen on the autoradiogram is very uniform for all parts of the brain as demonstrated in Fig. 4D. The increase in intensity of pulse-labeled components observed after the stage of puparium formation and the appearance of additional components is also uniform, but the maximum is at 60 hr rather than at the end of metamorphosis (refer to Fig. 4C).

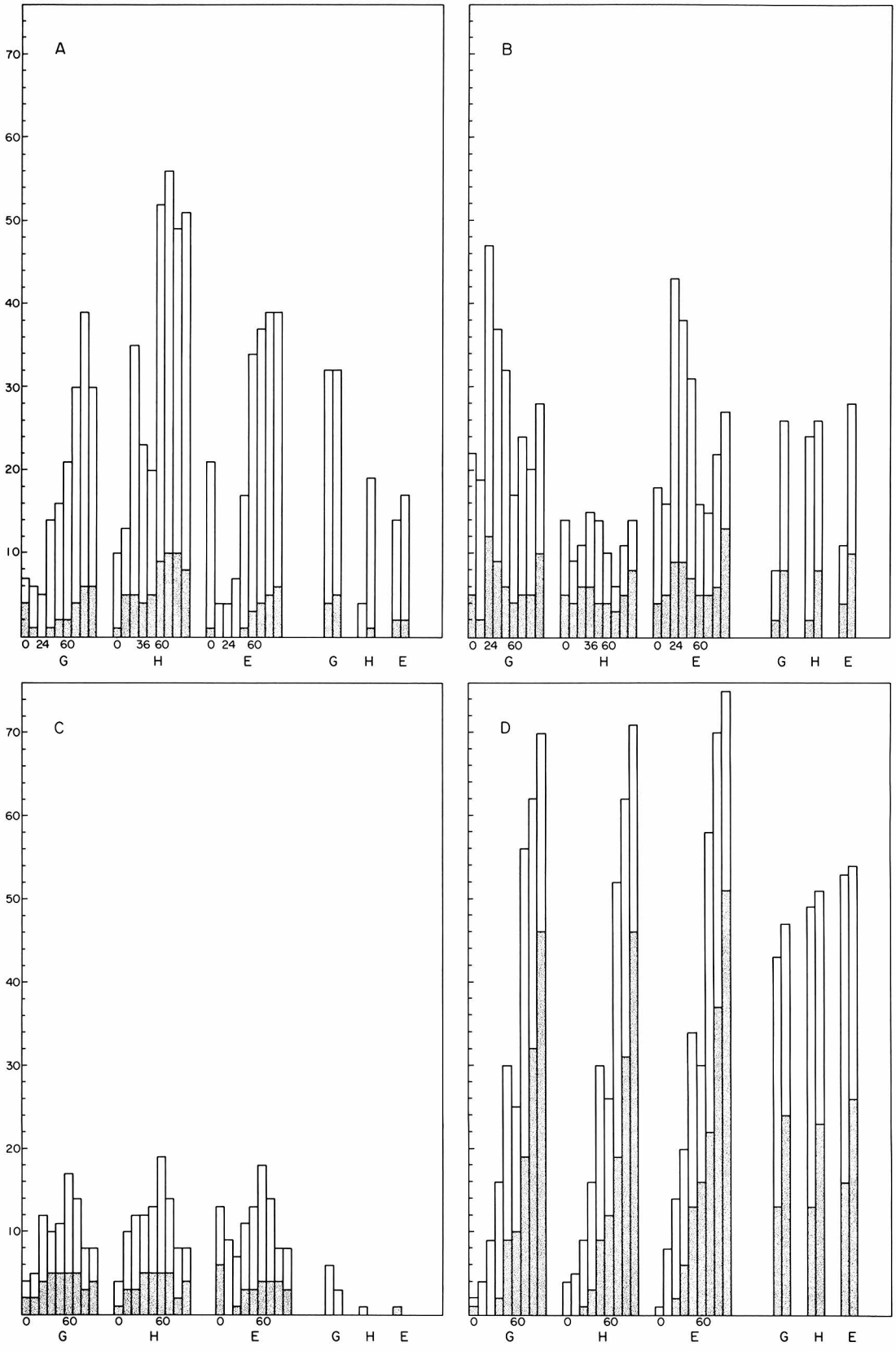
More detailed analysis of the total bulk of proteins shows that there are two major times in development at which the decrease in intensity or disappearance of some bands is observed. In the thoracic ganglion and the eye lobes, these periods occur at about 24 hr and after 72 hr, while in the hemispheres they are observed at 36 hr and after 72 hr (Fig. 4B). It is interesting that the time of fewest increases in intensity and fewest appearances of new bands occurs at 24 hr in thoracic ganglion and eye lobes and at 36 hr in hemispheres (Fig. 4A).

Figure 4. Histograms of changes in intensity and distribution for Coomassie-stained and ^{35}S-Met pulse-labeled components separated on 10-20% gradient SDS polyacrylamide gels.

The vertical axis indicates numbers of bands undergoing change and the horizontal axis indicates the developmental stage in hours after puparium formation. The initial white prepupal (t = 0) set of bands is compared with the larval pattern while all others are compared with the white prepupal set. On the right side of each histogram, the later stages are compared with the 72 hr pattern as well. The tissues are thoracic ganglion (G), hemispheres (H), and eye lobes (E).

(A) and (C) indicate increases in intensity of bands on Coomassie-stained and pulse-labeled patterns, respectively. The shaded portion represents those components which are newly appearing.

(B) and (D) indicate decreases in intensity of bands on Coomassie-stained and pulse-labeled patterns, respectively. The shaded portion represents those components which disappear completely.


A
B
C
D
70
60
50
40
30
20
10
0 24 60
0 36 60
0 60
G
H
E

The accumulation of the Coomassie-stained material towards the end of metamorphosis is well demonstrated in Fig. 6, where the patterns of two far-removed stages in development are compared. On the left of the figure in lanes 3 and 4 are aliquots of 7 brains from 5.5-7 hr prepupae and 89-91 hr pupae, respectively, and on the right are the parts of the brains of 12 and 84 hr pupae from a different gel. The arrows point to the major changes in intensities, most of which are increases. The new bands 19,300, 23,200, 24,800, 70,700 and for eye lobes, 33,700, occur at the end of metamorphosis and are very faint, so the observed general rise in intensity is actually due to increasing intensity of the previously existing bands.

Components specific to the parts of the brain. (Refer to Fig. 2A and B.) One striking feature of the protein content of the various parts of the brain is the remarkable similarity in distributions observed. There are only two bands (34,300 and 22,800) specific for the eye lobes which were detected on the Coomassie-stained gel. Both of these bands are very faint and are not visible on the autoradiogram of the same gel. Band 34,300 is already present in the larval stage. Its intensity starts to increase after 12 hr, reaches a maximum at 72 hr after puparium formation and remains at the same level up to the time of the emergence of the fly. Band 22,800 is observed only at the beginning of metamorphosis, up until 24 hr after puparium formation at which time it also appears in the hemispheres. No band specific for thoracic ganglion or hemispheres was detected on the Coomassie-stained pattern.

On the autoradiogram, pulse-labeled components specific to the eye lobes are of the following molecular weights: 78,500, 21,400, and 20,900. Again, all of these are present in very small, sometimes barely detectable amounts. Component 78,500 is observed at the white prepupal stage, the amount of it is

somewhat increased by pupation (12 hr) and it is not detected at all at 24 hr after puparium formation. However, on the Coomassie-stained pattern at the same position of migration, the band specific to the eye lobes is observed in the larval and prepupal stages, but starting from pupation it is present in thoracic ganglion and hemispheres as well and remains present in all until the end of metamorphosis. Component 21,400 is observed from 60 through 72 hr and is not detected at all in the Coomassie-stained pattern. Band 20,900 is present at white puparium formation (at the time of pupation, a band of the corresponding position is detected in the hemispheres) but is not seen again until 60 hr. At 72 hr, it is still present, but then disappears. On the Coomassie-stained pattern at the corresponding position, a band of equal intensity is observed starting from late larval stage up until 24 hr. By this time the intensity of the band has decreased in thoracic ganglion and the eye lobes, and by 60 hr the intensity of the band is the same for all parts again. The decrease in the intensity, however, continues with some slight variations for the parts, and, finally, by the time of emergence the band is no longer detected.

The remainder of the bands are present in all three parts and usually are of very similar if not identical intensity in the three parts. However, some variations do take place and occasionally a band temporarily disappears in only one part. A few bands which are striking because of high relative intensity in one particular part, and which persist over long periods of time, might be made note of. Such bands characteristic to the eye lobes are seen at the positions corresponding to 34,800, 17,800, 17,000, 16,500, and 15,400 (Fig. 2A). On the autoradiogram, these are present throughout metamorphosis with practically identical intensities in all parts of the brain except for the 16,500 band. This is uniquely detected in the eye lobes at late larval and white prepupal stages. By the time of pupation,

it slightly decreases in the eye lobes, but is already detected in the other parts and by 24 hr it has decreased even more and is present with equal intensity in all parts. After 24 hr, it disappears. Hemispheres and thoracic ganglion share two bands of such nature of molecular weights 33,700 and 50,500 daltons, which become of equal intensity in the eye lobes only after 72 hr and 36 hr, respectively. On the autoradiogram, both of the bands are of equal intensity in all parts during metamorphosis.

Glycoproteins. In order to determine whether the Coomassie-stained components have sugar moieties, three fluorescent lectins which bind to a wide variety of carbohydrates were used: RCA-60, known to recognize alpha- or beta-linked D-galactose and N-acetyl-D-galactosamine (22); Con A, known to recognize alpha-linked D-mannose or D-glucose at nonreducing termini and internal positions where sugar carbons 3, 4, 5 and 6 remain unlinked (23); and WGA, known to recognize alpha- or beta-linked N-acetyl-D-glucosamine and sialic acids in terminal positions (24-26). All three lectins showed very extensive binding, producing fluorescent patterns similar to each other and to the Coomassie pattern. The carbohydrate binding specificity of these lectins was checked by carrying out parallel incubations in the presence of hapten sugar inhibitors as described in Materials and Methods. The results of one such experiment, demonstrating Con A specific binding to carbohydrates, is shown in Fig. 5a and b.

One can count at least 75 fluorescent bands, and it is interesting to note that the intensity of the fluorescence is much stronger at the end of metamorphosis (Fig. 4a [lanes 1 and 2]). The changes in the fluorescence pattern and a comparison with the Coomassie pattern is shown on Fig. 6. Although one cannot make a direct superposition of the fluorescence and the Coomassie patterns (due to the differential swelling of the gradient polyacrylamide gel in the various solutions used for

Figure 5. Con A specific binding.

Electrophoretic patterns of D. melanogaster brain proteins and glycoproteins from a 10-20% exponential gradient polyacrylamide SDS gel.

(a) Incubated with fluorescein Con A which binds to alpha-linked D-mannose or D-glucose in their pyranose forms.

(b) Incubated with fluorescein Con A in presence of inhibitor alpha-methyl-D-mannopyranoside.

(c) and (d) Coomassie stained slices of a and b, respectively.

Lane 1 - 7 brains of 89-91 hr pupae.

Lane 2 - 7 brains of 5.5-7 hr prepupae.

Lane 3 - bovine γ-globin, ovalbumin, avidin, ribonuclease, and ovomucoid as standards, indicated bylines in descending order.

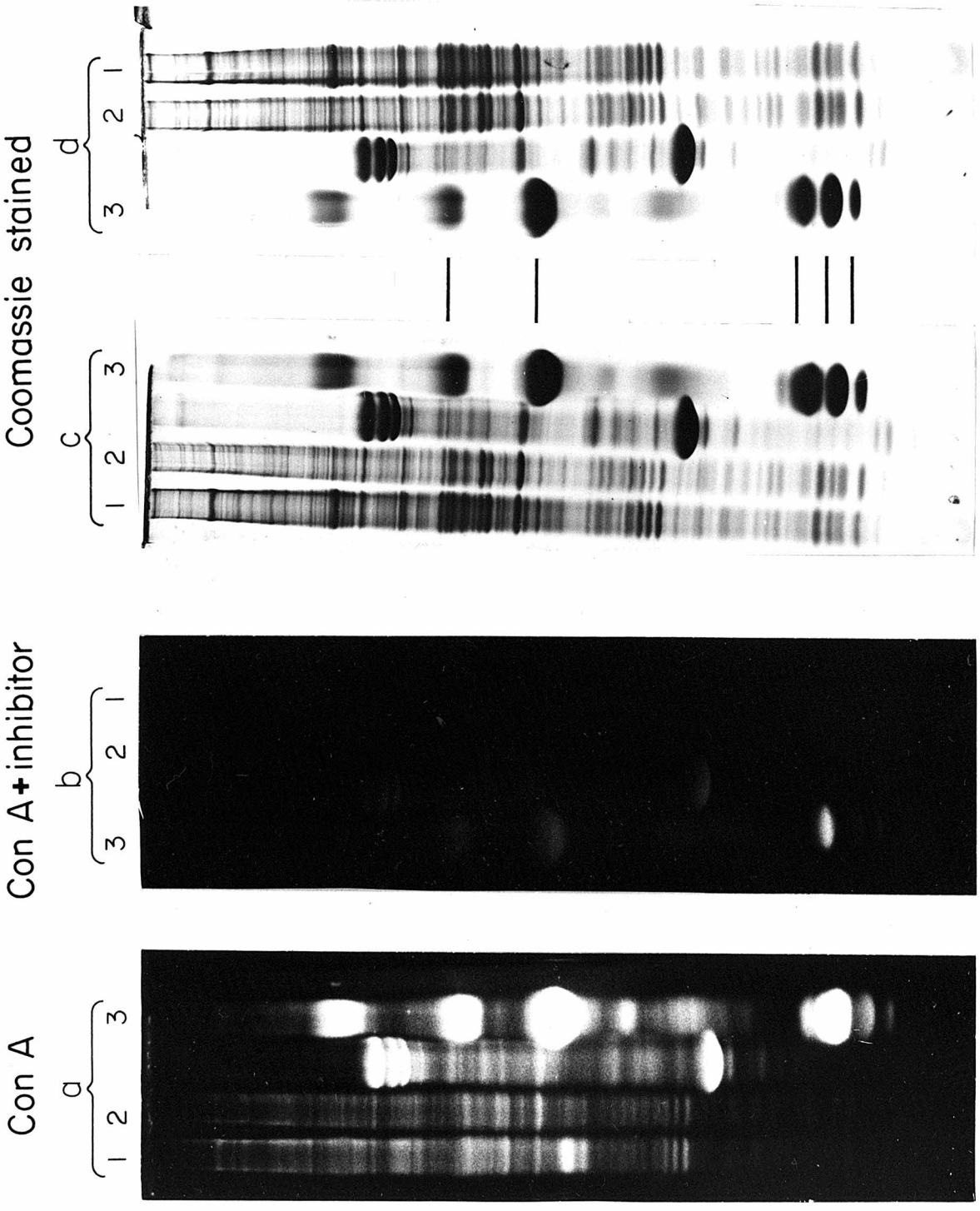
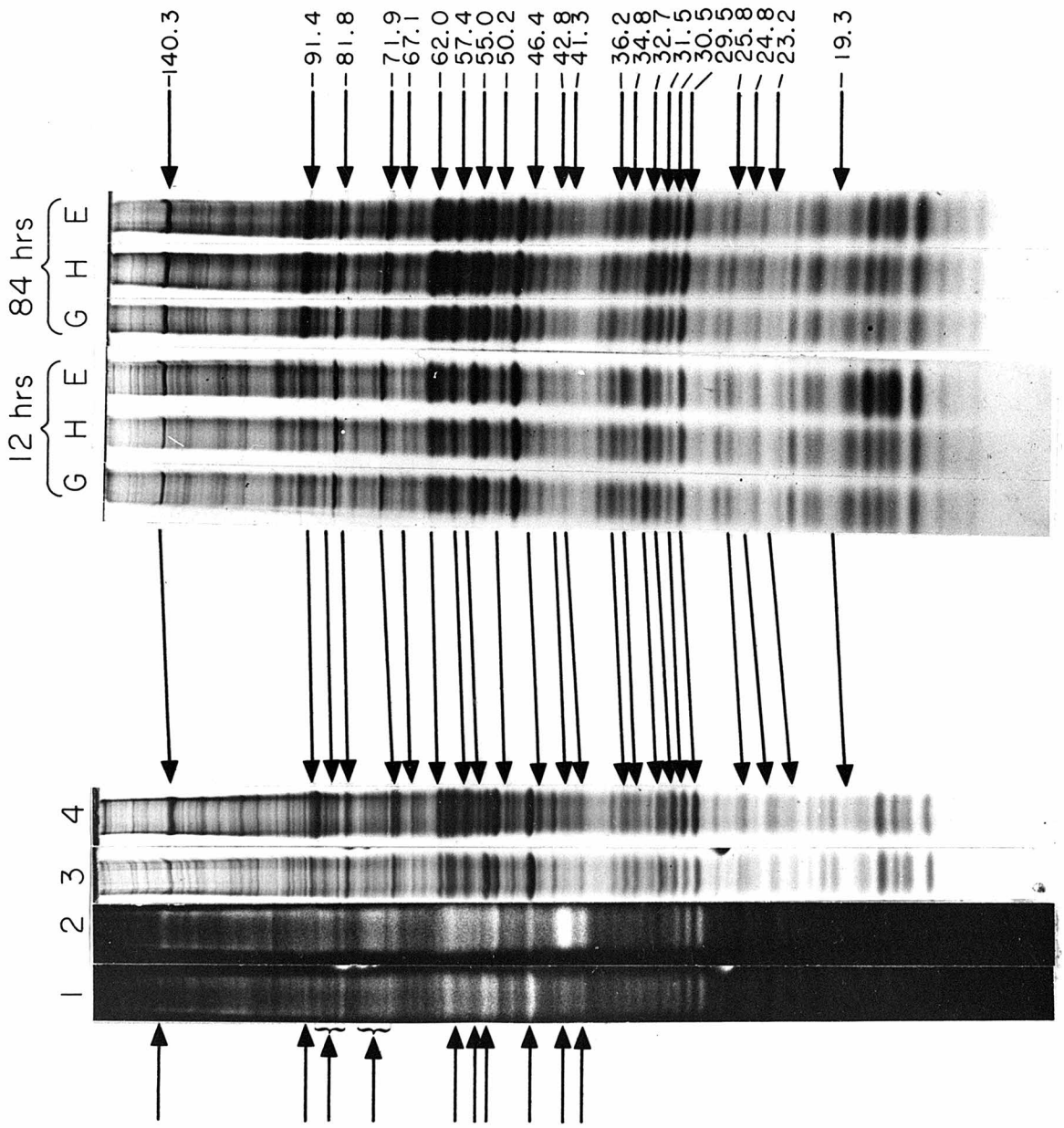

Coomassie stained
Con A+inhibitor
Con A
a
1 2 3
b
3 2 1
c
1 2 3
d
3 2 1

Figure 6. Comparison of electrophoretic patterns of D. melanogaster brains of two far-removed developmental stages.

On the Left: Lanes 1 and 3 show aliquots of 7 prepupal brains from 5.5-7 hr old animals at first bound with fluorescent Con A, and subsequently stained with Coomassie, respectively. Lanes 2 and 4 show aliquots of 7 pupal brains from 89-91 hr old animals at first bound with fluorescent Con A, and subsequently stained with Coomassie, respectively.

On the Right: Coomassie staining of proteins from parts of brains taken from 12 and 84 hr pupae. G - thoracic ganglion, H - hemisphere, E - eye brain. Brains from 12 animals were dissected for each stage.

Arrows point to the regions where the change of the pattern of intensity of the band takes place. MWts x 10^{-3} were determined as described in Materials and Methods.


140.3
91.4
81.8
71.9
67.1
62.0
57.4
55.0
50.2
46.4
42.8
41.3
36.2
34.8
32.7
31.5
30.5
29.5
25.8
24.8
23.2
19.3
84 hrs
G H E
12 hrs
G H E
4
3
2
1

photography of both patterns), it is still probable that the majority of the proteins are glycosylated, and the increase of the fluorescence intensity is associated rather with the accumulation of a component than with more extensive glycosylation of it.

There are many fewer bands in the fluorescence pattern than in the Coomassie-stained pattern, and consequently fewer changes are observed in the fluorescence pattern. This is most likely due to the fact that the fluorescein-lectin staining is less sensitive than Coomassie and therefore only relatively strong bands are detected.

DISCUSSION

The Drosophila brain is one of the few larval tissues which survives metamorphosis, or second embryogenesis, without hystolysis. The size of the brain does not change significantly during this period; compare panels D and F in Fig. 1. Also, we have observed that the general shape of the thoracic ganglion remains unchanged, although lateral constrictions in two areas form three discrete sections, and that the greatest change in morphology is observed with the hemispheres, which first divide to form distinct optic lobes and then partially refuse (see Fig. 1). The fact that "the rudiments of all regions of the adult optic lobes are already present in the third instar larva" as established by White and Kankel using pulse and pulse-chase labeling and thin sectioning techniques (27) is supported by the observation described here that the hemisphere of the late larval brain may be separated to reveal two distinct structures by gentle pulling of the eye disc from the hemisphere. In view of the morphological changes observed during the metamorphosis of the Drosophila brain, it is surprising that Coomassie-stained and pulse-labeled polypeptides show very little change over a 100-hr period (Fig. 2).

This lack of alteration in the brain contrasts sharply with numerous changes seen in other tissues. For example, Tissières et al. (5) noted that in the autoradiograms of salivary gland proteins, "of the great many bands which can be seen during the 16-hr period examined, one only, though varying in intensity, can be seen at all stages and five are visible at one stage only." Some of these bands appear for less than one hour (28). In the thoracic epithelium, which is formed only after puparium formation, many transiently existing bands are observed on both Coomassie-stained and autoradiogram patterns. These bands are highly stage specific (20, 21, Figure 4). Equally dramatic changes were observed for pulse-labeled band patterns in malpighian tubes (18) and flight muscles (19). It should be noted that the salivary glands, a tissue composed of secretory cells, are well-differentiated and not undergoing cell division during the period cited above. Thoracic epithelium, however, is an extensively differentiating tissue. Since both of these tissues show dramatic changes in polypeptide distribution during development, it was expected that the brain, which changes morphologically and contains neurosecretory cells, would show comparable changes.

In connection with the constancy of band patterns in brain tissue, a few words should be said about the pulse-labeling technique. The intensity of bands of the ^{35}S-Met pulse-labeled polypeptides on the autoradiogram is dependent on the methionine content of the polypeptides, the size of methionine pool, and the rate of synthesis. The latter two factors will govern changes in band intensity during development. The fact that different tissues which mature on a different time scale from brain [thoracic epithelium (3), flight muscles (19) and ovaries (29)] show similar decreases in ^{35}S-Met incorporation during development indicates that a change in the methionine pool is not responsible for the variation in ^{35}S-Met incorporation observed. Since the Coomassie-stained pattern remains fairly

constant, even towards the end of metamorphosis, it is unlikely that the decrease in intensities of the bands in the autoradiograms is due to an increased turnover rate. Therefore, these changes must reflect differences in the rate of polypeptide synthesis.

The long-lasting constant pattern of ^{35}S-Met-labeled peptides is expected to lead to accumulation of material, which is in fact observed (Figs. 2A, 4A, and 6). Since the rate of protein synthesis is rather constant until 48 hr and dramatically slows after that time for most of the bands (Figs. 2B and 4D), the decreases in band intensities of a subset of the total number of Coomassie-stained bands are dependent not on the rate of synthesis but on a differential rate of degradation of the polypeptides or possible transport of the material out of the brain. However, the latter seems to be unlikely since the decreases involve approximately one-third and sometimes even one-half of the total detectable number of components.

The observed accumulation of proteins in Drosophila brain (Figs. 2A and 6) combined with the observation of White and Kankel (27) that "the larval to adult transition during metamorphosis is accompanied by an increase in fiber mass and a pronounced change of cortical thickness," in the absence of hystolysis and with little if any cell division or change in cell type and size, lead us to the conclusion that the imago brain of Drosophila is formed mostly from larval cells, which during metamorphosis develop additional structures of types common to all parts of the central nervous system. This common cell structure might be extensive growth and/or reorganization of axons and dendrites. In this connection, it is interesting that the rise in intensity on the Coomassie-stained gel is due mainly to increasing intensities of pre-existing bands rather than to appearance of new bands (Fig. 6). The finding that most of the accumulating polypeptides are glycosylated (Fig. 5) supports the conclusion that the predominant change in the larval Drosophila brain

is dendritic and/or axonal reorganization, since these are highly membranous structures. A fascinating report on dendritic reorganization during metamorphosis of the Tobacco Hornworm moth by Truman and Reiss (30) lends some precedent to this explanation. These workers followed the development of a single neuron throughout metamorphosis and found a decrease in the size of the larval dendrite followed by formation of an adjacent dendrite and an increase in the sites of both dendrites.

Careful and thorough analysis of the Coomassie-stained gel (see Fig. 4B) shows a decrease in intensity after 72 hr which is consistent with the finding of White and Kankel that the 72-hr Drosophila brain is hystologically mature, whereas a decrease in intensity at 24 and 36 hr can be explained by extensive degradation of larval dendrites and axons at that time.

There is a striking similarity in polypeptide distribution for all examined parts of the nervous system. In the optic lobes, which contain the greatest number of unique components, two such bands are present on the Coomassie-stained gel and three on the autoradiogram. All of these are faint and two of the five are visible only at the beginning of metamorphosis. In addition, one band present in all parts examined is more intense on the Coomassie-stained gel and autoradiogram for the optic lobes in the beginning of metamorphosis but decreases in intensity on the autoradiogram after the time of pupation, becoming similar in intensity with other brain parts. The uniform distribution of polypeptides in all brain parts is consistent with the conclusion that a reorganization of dendrites and axons, common to all brain parts, is the primary mode of development in the Drosophila brain during metamorphosis. The appearance of unique bands in the optic lobes at the beginning of metamorphosis agrees with the observation of White and Kankel that cellular redistribution to form the lamina, medula and lobula is

complete by the time of pupation (30). It is probable that many more unique polypeptides exist during this developmental period but are not observed due to the limitations of the technique. Presumably, 1000 polypeptides can be resolved on two-dimensional isoelectric focusing-SDS electrophoresis gels (31). In contrast, about 100 polypeptides have been observed in this work on the Drosophila brain. It is possible, then, that only a small fraction of the actual component polypeptides have been observed.

The proposed reorganization of larval neurons prompts one to consider which proteins are involved in determination of cellular morphology and movement. Actin is involved in movement of cells and cellular components (32) and, along with tubulin, is important in cell differentiation (33, 34). Microtubules, the structures formed from tubulin, are especially prominent in neural development; the highly asymmetric neural processes are packed with dense parallel arrays of microtubules. Recently, Spiegelman et al. (35) have demonstrated that the aggregation of microtubule initiation sites is preceding neurite outgrowth in mouse neuroblastoma cells. Therefore, one would expect that if dendritic reorganization is an important process in Drosophila brain, tubulin and actin should be especially prominent among the proteins. Preliminary experiments show that sea urchin tubulin (α and β chains) and actin from chick skeletal muscle comigrate with the most pronounced bands (of molecular weights 50,500, 49,600 and 47,800, respectively) of Drosophila brains.

The Drosophila brain is of interest with regard to the question of whether the brain controls the development of other tissues or vice versa. Evidence is accumulating which bears on this important question. Meyerowitz and Kankel report that the Drosophila eye disc influences the development of the lamina and medula of the optic lobe (36). Also, the lack of sensory fibers from the halters

and wings and/or the imaginal disc precursors of these structures results in a much less elaborated shape of the thoracic ganglion (37). In light of the fact that nerve growth factor, which promotes microtubule formation, is essential for normal development and maintenance of neural tissue (38), it is possible that this or a similar factor, which may be produced in other tissues, governs the dendritic reorganization by which the Drosophila brain develops.

REFERENCES

1. Nordlander, R. H. and Edwards, J. S. (1969). W. Roux' Archiv. **163**, 197-220.
2. Mitchell, H. K. and Mitchell, A. (1964). Drosophila Inf. Serv. **39**, 135-137.
3. Mitchell, H. K. and Lipps, L. S. (1977). Biochem. Genet. **15**, 575-587.
4. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971). Biochemistry **10**, 2606-2617.
5. Tissières, A., Mitchell, H. K., and Tracy, U. M. (1974). J. Mol. Biol. **84**, 389-398.
6. Laemmli, U. K. (1970). Nature (Lond.) **227**, 680-685.
7. Van Blerkom, J. and Manes, C. (1974). Dev. Biol. **40**, 40-51.
8. Mirault, M.-E., Goldschmidt-Clermont, J., Moran, L., Arrigo, A. P., and Tissières, A. (1977). Cold Spring Harbor Symp. Quant. Biol. **42**, 819-827.
9. Nolan, C. and Smith, E. L. (1962a). J. Biol. Chem. **237**, 446-452.
10. Nolan, C. and Smith, E. L. (1962b). J. Biol. Chem. **237**, 453-458.
11. Anderson, B. R. (1969). Immunochemistry **6**, 739-749.
12. Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., and Kobata, A. (1975). J. Biol. Chem. **250**, 8569-8575.
13. Plummer, T. H., Jr. and Hirs, C. H. W. (1964). J. Biol. Chem. **239**, 2350-2358.
14. Fournet, B., Leroy, Y., and Montreuil, T. (1974). Colloques Internationaux du Centre National de la Recherche Scientifique No. 221 Villeneuve D'ASCQ 20-27 juin 1973, pp. 111-130.
15. Bayard, B. and Montreuil, T. (1974). Colloques Internationaux du Centre National de la Recherche Scientifique No. 221 Villeneuve D'ASCQ 20-27 juin 1973, pp. 209-218.
16. West, C. M., McMahon, D., and Molday, R. S. (1978). J. Biol. Chem. **253**, 1716-1724.

17. Karnovsky, M. T. (1965). Cell Biol. **27**, 137A.

18. Moller, G. Unpublished result.

19. Moller, G., Petersen, N. S., and Mitchell, H. K. Unpublished result.

20. Mitchell, H. K., Lipps, L. S., and Tracy, U. M. (1977). Biochem. Genet. **15**, 575-587.

21. Chomyn, A., Moller, G., and Mitchell, H. K. (1979). Dev. Genet. In press.

22. Nicolson, G. L. and Blaustein, J. (1972). Biochim. Biophys. Acta **266**, 543-547.

23. So, L. L. and Goldstein, I. J. (1968). J. Biol. Chem. **243**, 2003-2007.

24. Greenaway, P. J. and Levine, D. (1973). Nature New Biol. **241**, 191-192.

25. Allen, A. K., Neuberger, A., and Sharon, N. (1973). Biochem. J. **131**, 155-162.

26. Goldstein, I. J., Hammarstrom, S., and Sundblad, G. (1975). Biochim. Biophys. Acta **405**, 53-61.

27. White, K. and Kankel, D. R. (1978). Dev. Biol. **65**, 296-321.

28. Mitchell, H. K. Unpublished result.

29. Petersen, N. S. and Moller, G. Unpublished observations.

30. Truman, J. W. and Reiss, S. E. (1976). Science **192**, 477-479.

31. O'Farrel, P. (1975). J. Biol. Chem. **250**, 4007-4021.

32. Pollard, T. D. and Weihing, R. R. (1974). CRC Critical Rev. Biochem. **2**, 1-64.

33. Perry, M. M. and Waddington, C. H. (1966). J. Embryol. Exp. Morphol. **15**, 317-330.

34. Granholm, N. H. and Baker, J. R. (1970). Dev. Biol. **23**, 563-584.

35. Spiegelman, B. M., Lopata, M. A., and Kirschner, M. W. (1979). Cell **16**, 253-263.

36. Meyerowitz, E. M. and Kankel, D. R. (1978). Dev. Biol. **62**, 112-142.

37. Furrús, A. Personal communication.

38. Campenot, R. B. (1977). Proc. Natl. Acad. Sci. USA **74**, 4516-4519.

CHAPTER II

Patterns of Protein Synthesis Following Heat Shock in Pupae of *Drosophila melanogaster*

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ABSTRACT

Pupae of Drosophila melanogaster were heat shocked under conditions required to induce phenocopies in more than 90% of the flies that subsequently emerge. The effects of these treatments on protein synthesis in two tissues (thoracic epithelium and brain) were followed for several hours after the heat treatments. Results from pulse labeling and protein separations on SDS acrylamide gels showed a virtually complete cessation of protein synthesis immediately after the shock followed by a non-coordinate resumption of the starting pattern. Similar experiments following double heat shocks demonstrated a more rapid resumption of synthesis of heat shock proteins after two successive heat treatments than after a single one.

KEY WORDS

Drosophila melanogaster; pupae; heat shock; protein synthesis; phenocopies.

INTRODUCTION

A heat shock of 37.5°C for 20 min induces the synthesis of at least 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 of polytene chromosomes with in vivo labeled RNA from heat shocked cells (4, 5) taken together with results from in vitro translation experiments using polysomal RNA from heat treated cells (6, 7) have shown that these 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). Moreover, some of the recombinant DNAs containing sequences complementary to 87A and 87B inhibit, by hybridization arrest, the synthesis of one of the major heat shock proteins in an in vitro translation system (10). The heat shock phenomena in Drosophila provide, therefore, the basis for a suitable system for studying regulation of gene expression in eukaryotic cells.

In order to obtain more information toward understanding the events involved in the induction of synthesis of heat shock proteins and resumption of normal protein synthesis, and also toward understanding the molecular basis of phenocopy production, we have analyzed the effects of heat treatment under the conditions used earlier for phenocopy production (11-15). This temperature

treatment (40.2°C for 40 min), but not that at 37.5°C for 20 min, produces stage-specific phenocopies, many of which are similar to known mutants. 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 (15) 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.

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 (16). 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 (17).

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 (18).

The animals were synchronized by flotation at 5 hr after puparium formation (18). 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 (16). 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 (3-[N-morpholino]propanesulfonic acid) buffered medium (16) containing about 20 µCi of ^{35}S-methionine of specific activity 570 Ci/mMole (New England Nuclear). After a 25 min incubation at 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. (19) 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 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 as described earlier (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, 17). The SDS-containing buffers of Laemmli (20) were used. Staining and destaining were carried out essentially as described by Fairbanks et al. (19). The gels were dried and exposed for autoradiography on Kodak SB-5 no screen X-ray film. Autoradiogram exposures were usually for 1 to 2 days. The autoradiogram from which most of the lanes in Figure 4b were taken was exposed for 30 days.

Phenocopies

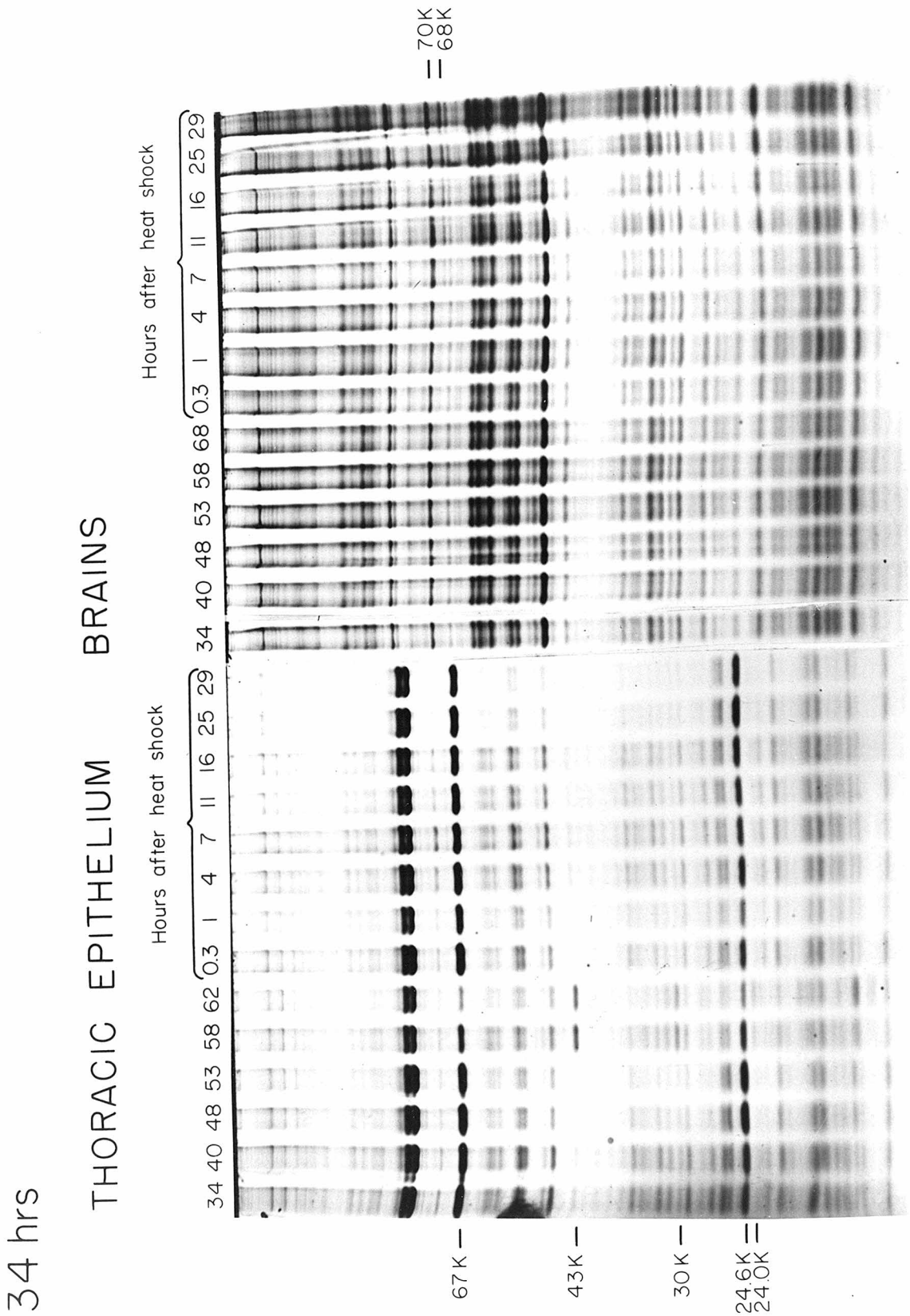
Animals from the same heat shocked populations used for the dissections were allowed to develop at 25°C and the adults examined for heat-induced phenotypes in the dissecting, phase contrast, or scanning electron microscope (15).

RESULTS

Thoracic epithelial tissue and brain were dissected at various ages 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 25°C. Figures 1, 2, and 3 show the electrophoretic patterns (Coomassie blue

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 (21) based on these values was used to determine the molecular weights of the other proteins.


34 hrs
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
70K
68K
67K
43K
30K
24.6K
24.0K

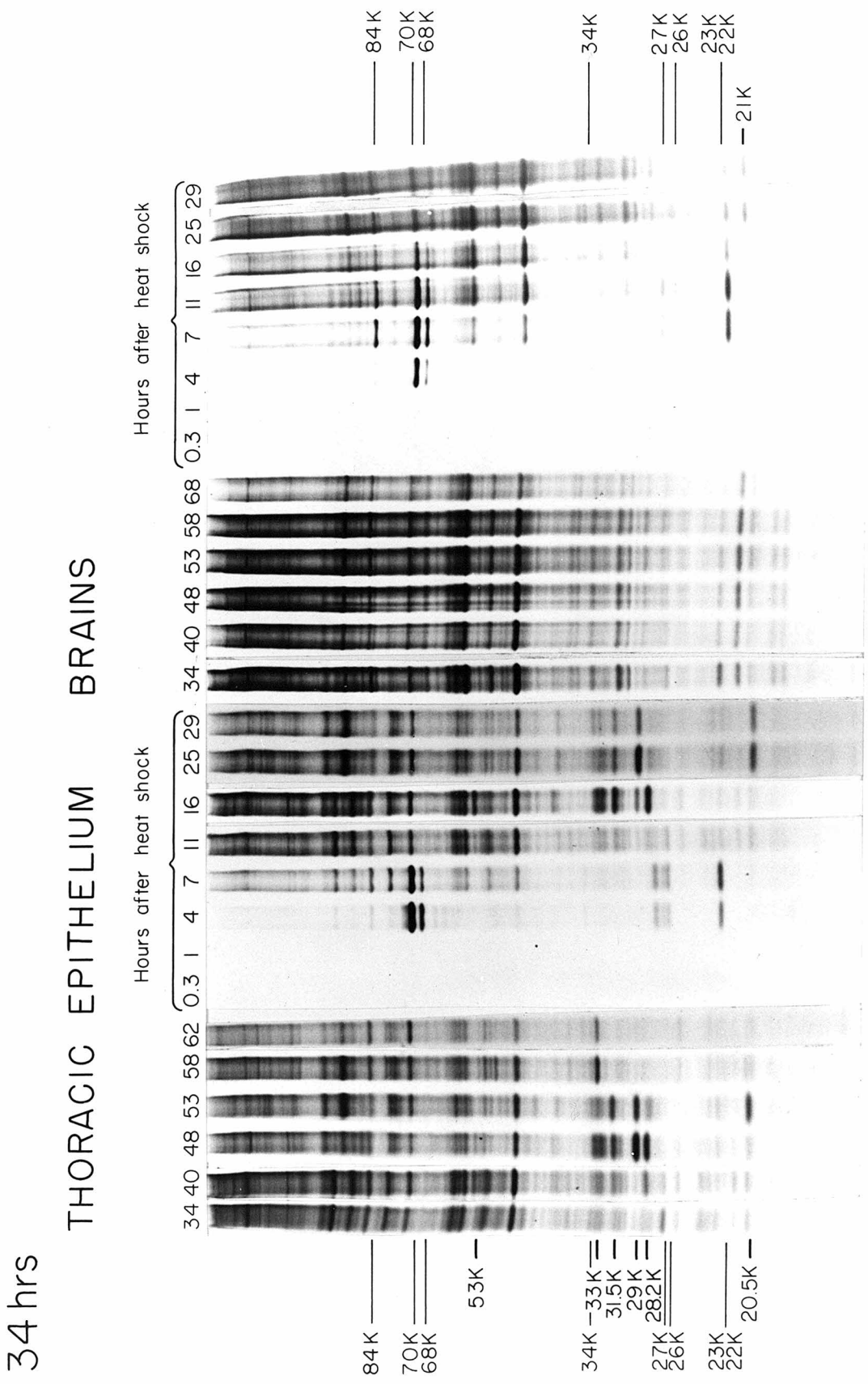
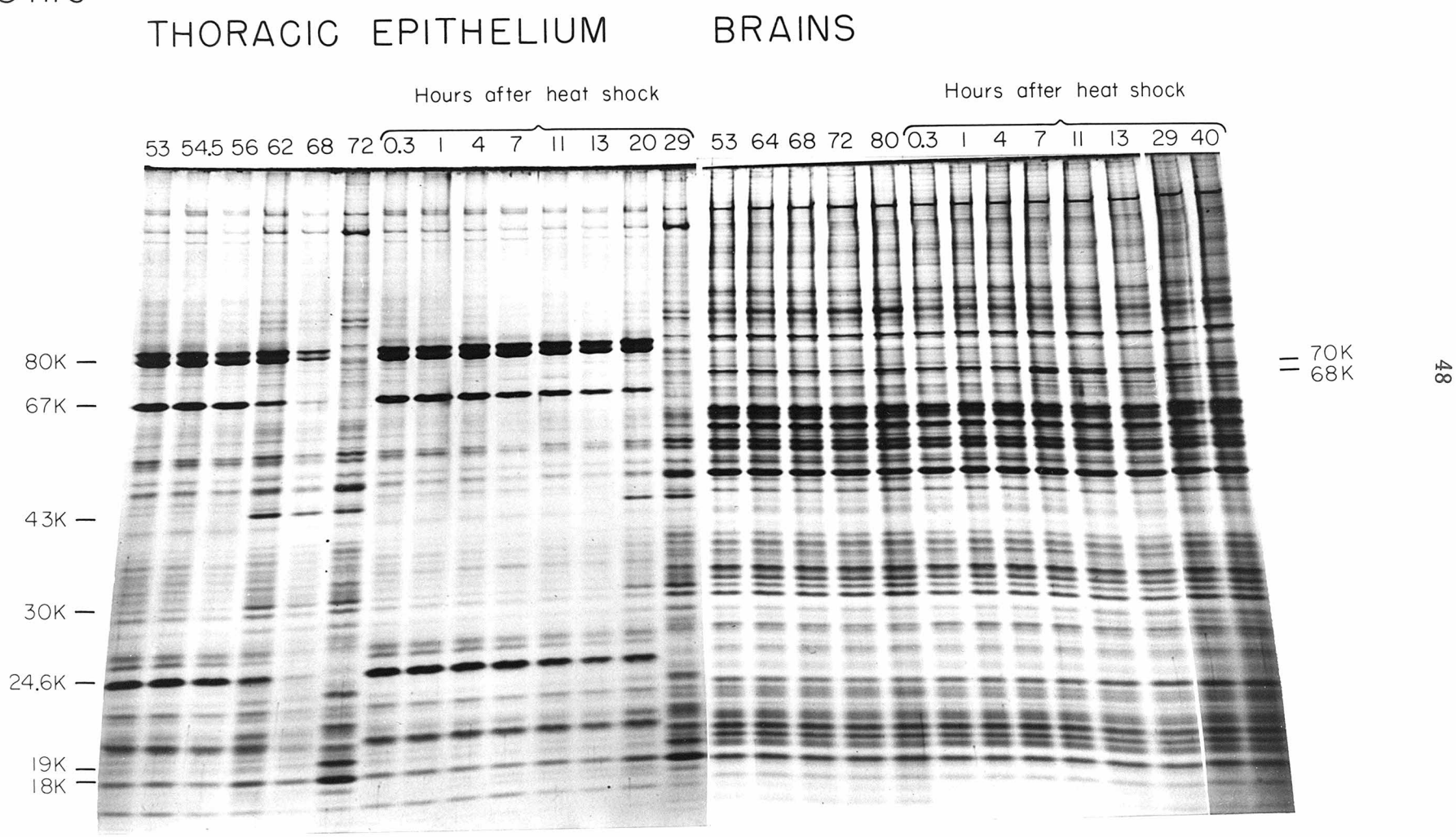

34 hrs
THORACIC EPITHELIUM BRAINS
Hours after heat shock
0.3 1 4 7 11 16 25 29
34 40 48 53 58 62
Hours after heat shock
0.3 1 4 7 11 16 25 29
34 40 48 53 58 68
84K
70K
68K
53K
34K
33K
31.5K
29K
28.2K
27K
26K
23K
22K
20.5K
84K
70K
68K
34K
27K
26K
23K
22K
21K

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


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

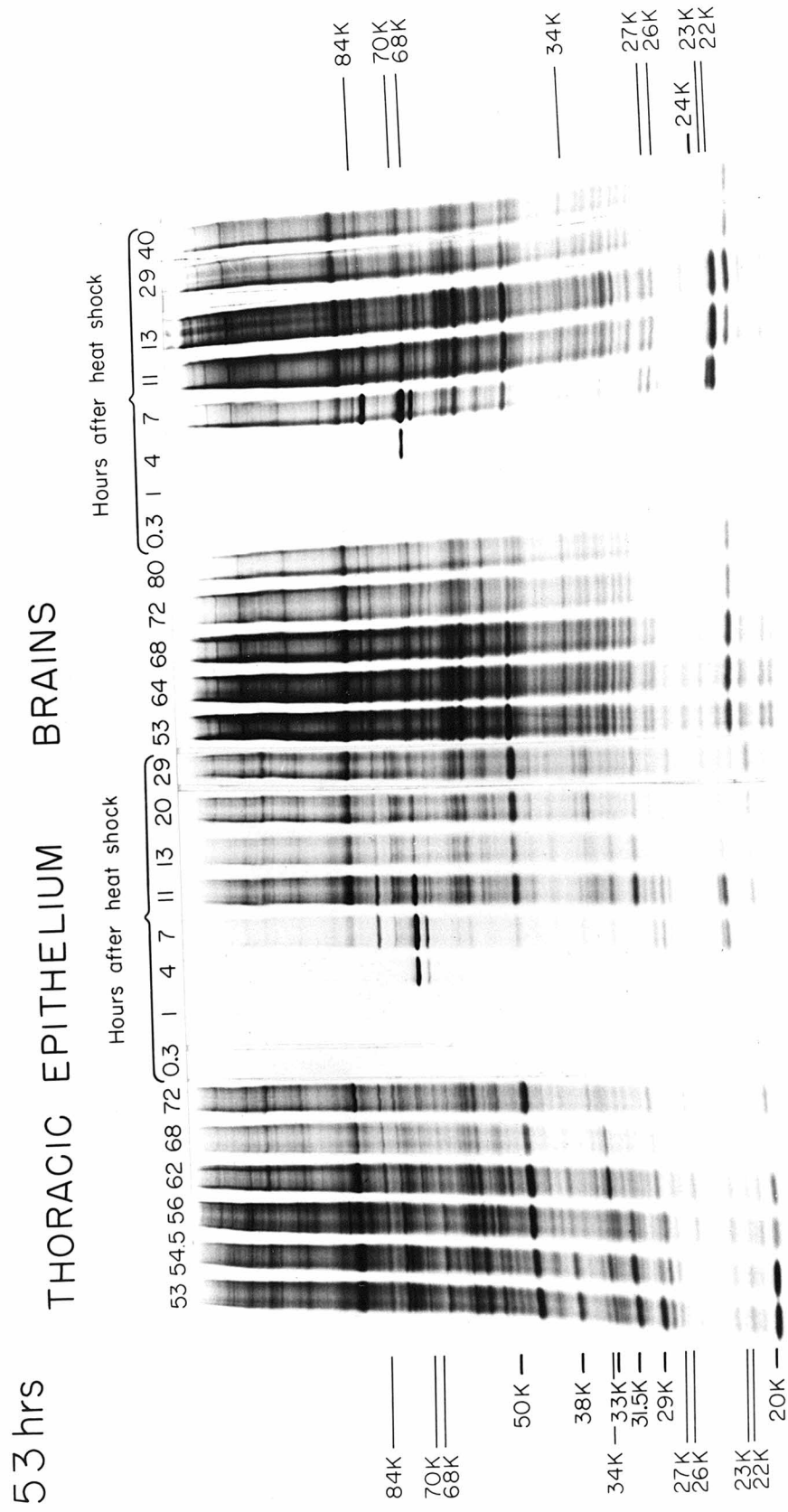
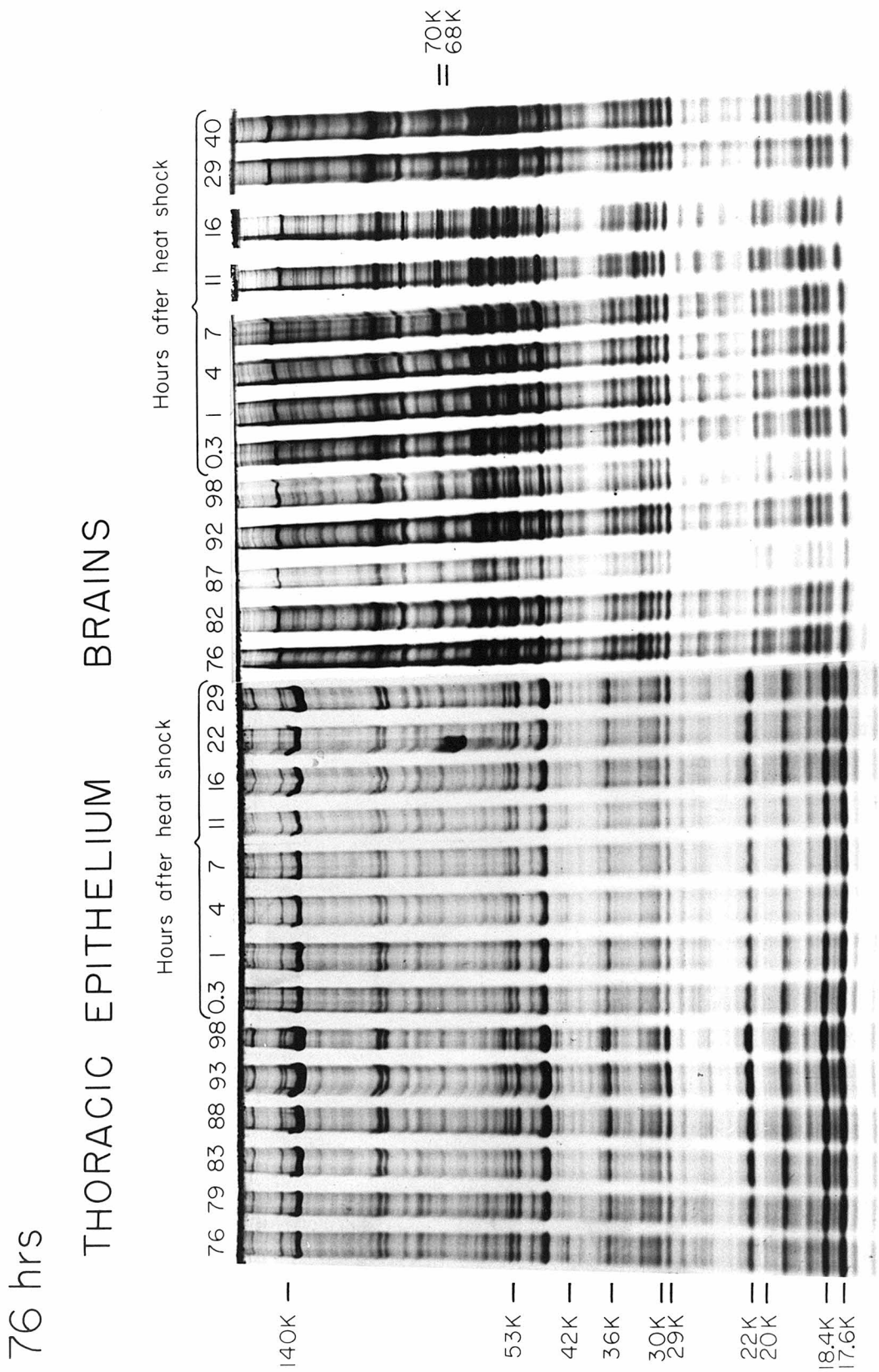
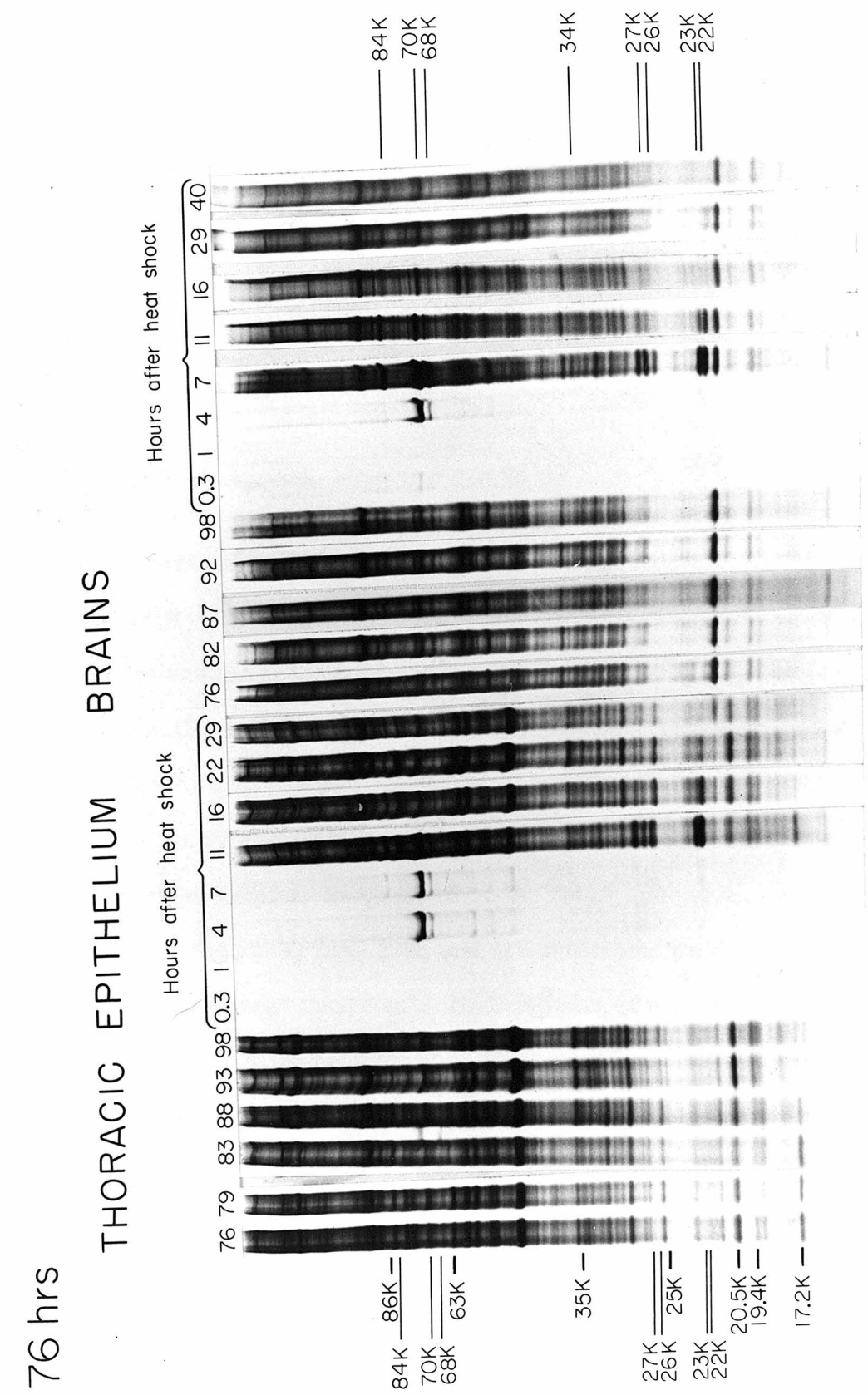

53 hrs
THORACIC EPITHELIUM
BRAINS
Hours after heat shock
53 54.5 56 62 68 72 0.3 1 4 7 11 13 20 29 53 64 68 72 80 0.3 1 4 7 11 13 29 40
84K
70K
68K
50K
38K
34K
33K
31.5K
29K
27K
26K
23K
22K
20K
17K
84K
70K
68K
34K
27K
26K
24K
23K
22K

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
THORACIC EPITHELIUM BRAINS
Hours after heat shock
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
70K
68K
140K
53K
42K
36K
30K
29K
22K
20K
18.4K
17.6K


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

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 drastically decreased (Figs. 1b, 2b, 3b). From densitometer tracings of the autoradiograms it was 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. Their labeling is maximal around 7 hr or between 7 and 11 hr after heat shock in all cases except in the thoracic epithelium from pupae heat shocked at 34 hr, where the labeling is maximal between 4 and 7 hr after heat treatment (Fig. 1b). At about the same time that the labeling 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. Since several of the heat shock proteins have mobilities coinciding with those of normal proteins in brain and thoracic epithelium, the exact onset and cessation of heat induced synthesis cannot be clearly defined.

b) Double heat shock

Figure 1b shows that after a single heat shock to 34 hr pupae, maximum rates of 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).

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 marked decrease in the rate of heat shock protein synthesis before resuming a 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

a) Single heat shock

The thoracic epithelial protein synthesis patterns exhibit many changes during development (16) 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 (17) 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 shown in Tables 1, 2, and 3. 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 at specific times following 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 thoracic epithelium. In pupae which were heat shocked at 34 hr, this protein appears 16 hr after the shock, i.e., at 51 hr after puparium formation, but is not yet detectable at 11 hr after the shock, i.e., 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 following heat shock at 53 hr. The thoracic epithelial tissue synthesizes a 50K dalton protein in 62 hr pupae and to a lesser

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	+	+	+	+	-	-	+	+	+	+
25K	-	-	-	-	+	+	-	-	±	-
20.5K	+	+	+	+	++	++	+	++	++	+
19.4K	+	+	+	+	++	++	++	++	++	+
18.8K	+	+	+	+	±	±	+	+	+	+
17.2K	++	++	++	+	±	±	++	±	±	±

extent, in 68 hr pupae (Fig. 2b, Table 2). In pupae heat treated at 53 hr, this protein is labeled at 20 hr after shock, at 74 hr after puparium formation, but not at 13 hr after the shock, i.e., at 67 hr. In addition, a 33K protein, labeled in 62 hr and 68 hr pupae is also labeled in 74 hr and 83 hr heat shocked pupae but not in 67 hr pupae. Thus, a developmental delay of at least 5 hr is indicated with respect to the synthesis of both the 50K and 33K dalton proteins.

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 strongly labeled, to the same degree, at 53 hr, and the latter protein intensely labeled 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 again seen as intensely labeled as the 29K protein.

A 20K protein, also highly labeled 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 may have occurred between 7 and 11 hr after the shock, and may, therefore, not have been observed.

The autoradiograph of the brain samples (Fig. 2b) shows changes clearly 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. These 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. Specifically, the relative intensities of the proteins of molecular weight 86K, 25K, 20.5K, 19.4K, and 18.8K are never the same with respect to one another in thoracic epithelium from heat treated pupae as from normal pupae.

b) 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.

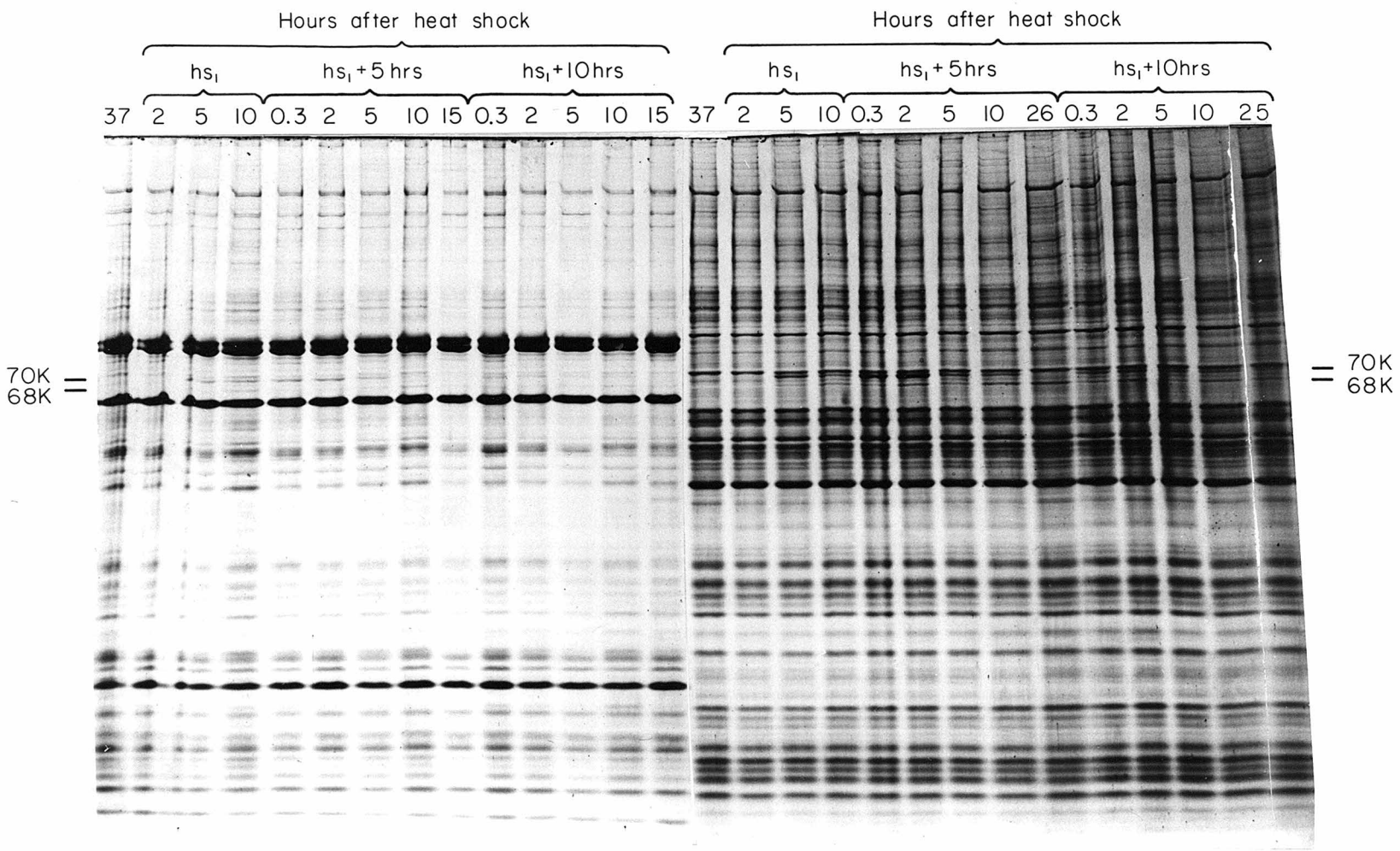
Coomassie stained patterns

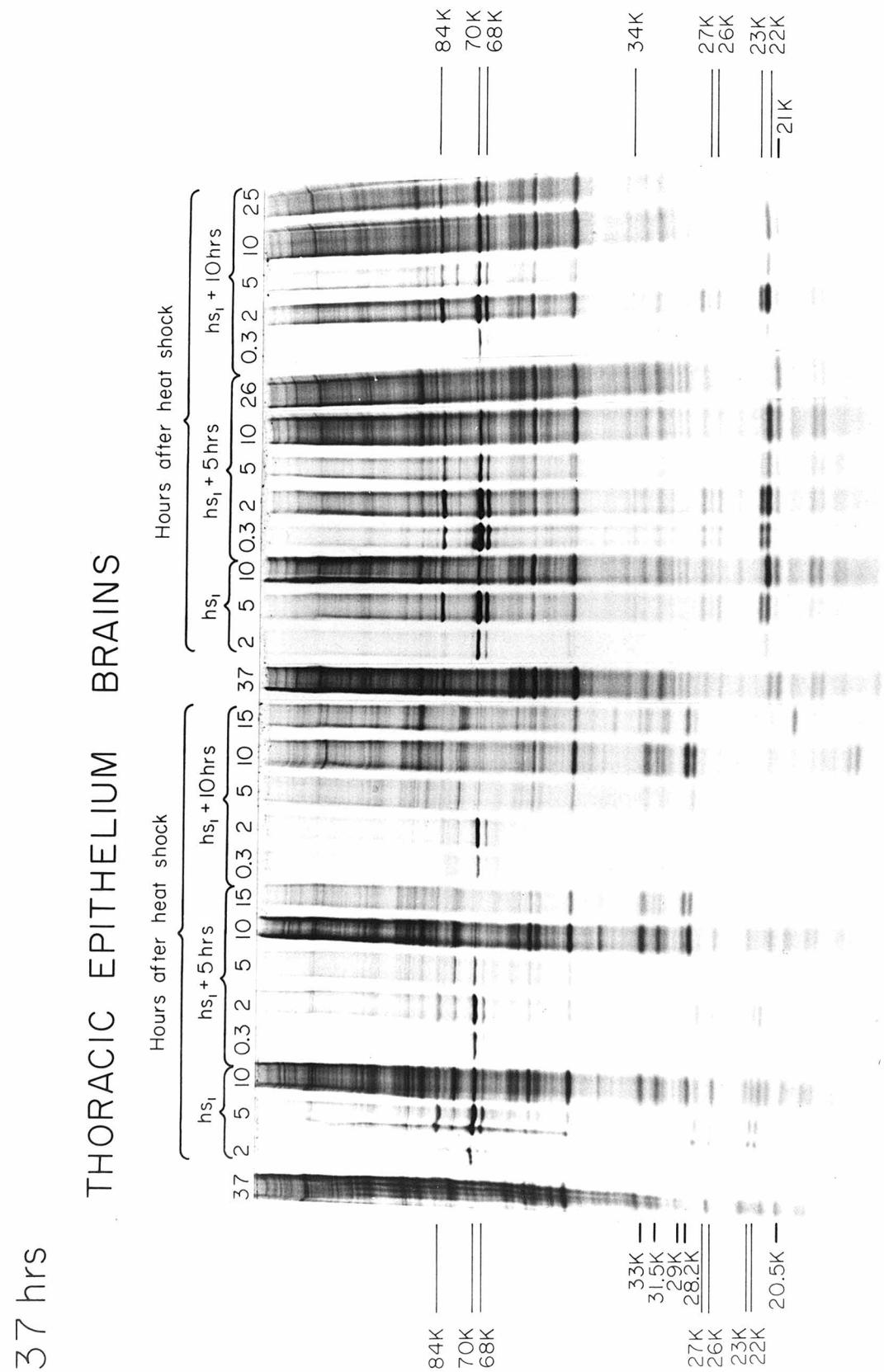
Figures 1a, 2a, and 3a show that heat treatment affects, in addition to the protein synthesis patterns, the steady state amounts of proteins which are in concentrations high enough to be detected by staining of the gel. At 58 hr, a 67K protein in thoracic epithelial cells begins to decrease in concentration 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 the shock (at age 64 hr) (Fig. 1a), while pupae treated at 53 hr begin to lose this protein at perhaps 13 hr after the treatment (at 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 the shock (Fig. 1a). Pupae heat shocked at 53 hr show accumulation of this protein at 20 hr but not at 13 hr after heat shock (at ages 74 hr and 67 hr, respectively) (Fig. 2a). Two other proteins which increase and decrease in

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.

37 hrs

THORACIC EPITHELIUM BRAINS




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

concentration at about the same time as the above mentioned changes are the 30K and 24.6K molecular weight proteins, respectively.

A delay in the change in the steady state amounts of proteins in heat shocked animals is also evident in pupae treated at 76 hr. More specifically, the change with time in the relative intensities of a 53K protein and one migrating just behind it is delayed by at least 13 hr in the heat treated animals (Fig. 3a).

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, the intensities 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 (15). 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 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

The experiments described in this paper were designed to evaluate the effects of heat shock on protein synthesis in different tissues of Drosophila under

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.

conditions which induce phenocopy production (11-15). In general such conditions are considerably more drastic than those used to induce production of heat shock proteins (1-4, 6, 7, 22) and they border on lethality in terms of temperature, time of exposure, or both.

Heat shock response in brain and epithelium

The brain of Drosophila is one of the few larval tissues which survives metamorphosis and it might therefore be expected to undergo relatively little change during development. Epithelial tissue, on the other hand, is formed anew after puparium formation and extensive differentiation occurs during the pupal stages. It was with these fundamental differences in origins in mind that brain and thoracic epithelium were selected as tissues to be used for a comparison of heat shock effects on protein synthesis. That the two tissues are indeed very different is shown clearly by the protein synthesis pattern in Figures 1b, 2b, and 3b. That is, for example, in Figure 1b the pulse-label patterns at the left covering the time period of 34 to 62 hr for epithelium show many rapid changes with time while the corresponding series for brains (34 to 68 hr) show nearly constant patterns. Similar relations hold for the series beginning at 53 hr (Fig. 2b) and 76 hr (Fig. 3b). However it is important to note that fewer and fewer changes occur in the pulse-label patterns from epithelium as the tissue matures.

Considering now the heat shock effects on these two kinds of tissues at three developmental stages it is clear that the most drastic changes are very similar regardless of the stage or tissue. That is, a shock of 40.2°C for 40 min results in a radical decrease in translation in all cases. This persists for more than 1 hr and it is followed by a production of heat shock proteins only, at 4 hr, and a resumption of general protein synthesis between 7 and 11 hr. Even at 11 hr,

recovery is not complete. Thus, in a gross sense the heat shock response in brain and epithelium is quite similar. This conclusion applies to the induction of a delay in all protein synthesis, to the induction of a set of specific heat shock proteins, and to the slow recovery of a normal pattern of proteins that are synthesized. It is in this last situation where differences in heat shock effects become evident. That is, when all protein synthesis is shut down abnormalities in the recovery process are more evident in the less mature (or more changing) tissues.

Heat shock and phenocopies

Much of the work that has been done on heat shock effects on protein synthesis in Drosophila (1-3, 22) or Drosophila cell cultures (4-7) was carried out at 37 or 37.5°C. Under these conditions total protein synthesis in 34 hr pupae is reduced by no more than 50% in brain and no more than 80% in epithelial tissue. Furthermore, protein synthesis resumes almost at once at 25°C immediately following these heat treatments.

In contrast to these observations, the exposure of animals to 40.2°C for 40 min, as carried out in the present experiments, shuts down all protein synthesis to less than 5% and it produces phenocopies to a level of 100% in pupae at specific stages (15). It seems apparent then that the severe repression of normal protein synthesis that is produced by the higher temperature treatment is an essential prelude to phenocopy production. An obvious possible mechanism for phenocopy formation rests on a non-coordinated recovery of a normal protein synthesis pattern and some such examples have been cited here.

Delay in development caused by heat shock

The approximately 5 hr to 10 hr delay in the sequence of normal changes

in the thoracic tissue may reflect a similar delay in development, and in fact, this is substantiated for the 5 hr by two facts reported here and elsewhere. Mitchell and Lipps (15) 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 characteristic of the first treatment 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 shock. Lindsley and Poodry (23) have also reported developmental delays caused by heat shock in pupae.

Heat shock protein synthesis and 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 the shock (Fig. 4b), as compared to 5 hr after a single shock. Yet, the resumption of normal protein synthesis occurs at the same time after the double shock as after a single shock, i.e., 7 to 11 hr after the shock. Thus, the return to normal protein synthesis is not tightly coupled to heat shock protein synthesis.

At 5 hr after a heat treatment, the tissues are synthesizing primarily heat shock proteins, and if a second treatment is given at this time, it is not surprising that the heat shock proteins are again strongly labeled only 2 hr after the shock. In brains, there is no marked decrease in protein synthesis after the second shock. Thus, in this case, heat shock translation in progress is resistant to the usual damping

influence that a heat shock has on protein synthesis.

In the case of a second shock given 10 hr after the first, heat shock protein synthesis may be reinduced faster than after a single 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 a 10 hr interval double shock, which may be due to some repeated impairment of metabolic function or of translation.

ACKNOWLEDGEMENTS

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REFERENCES

1. Tissieres A, Mitchell HK, Tracy UM (1974): Protein synthesis in salivary glands of Drosophila melanogaster: Relation to chromosome puffs. J Mol Biol 84:389-398.
2. Lewis M, Helmsing PJ, Ashburner M (1975): Parallel changes in puffing activity and patterns of protein synthesis in salivary glands of Drosophila. Proc Nat Acad Sci 72:3604-3608.
3. Moller G, unpublished.
4. McKenzie SL, Henikoff S, Meselson M (1975): Localization of RNA from heat induced polysomes at puff sites in Drosophila melanogaster. Proc Nat Acad Sci 72:1117-1121.
5. Spradling A, Pardue ML, Penman S (1977): Messenger RNA in heat shocked Drosophila cells. J Mol Biol 109:559-587.
6. Mirault ME, Goldschmidt-Clermont M, Moran L, Arrigo AP, Tissieres A (1977): The effect of heat shock on gene expression in Drosophila melanogaster. Cold Spring Harbor Symp Quant Biol 42:819-827.
7. McKenzie SL, Meselson M (1977): Translation in vitro of Drosophila heat shock messages. J Mol Biol 117:279-283.
8. Ritossa FM (1963): New puffs induced by temperature shock, DNP and salicylate in salivary chromosomes of D. melanogaster. Drosophila Inf Serv 37:122-123.
9. Ashburner M (1970): Patterns of puffing activity in the salivary gland chromosomes of Drosophila. V. Responses to environmental treatments. Chromosoma 31:356-376.

10. Schedl P, Artavanis-Tsakonas S, Steward R, Gehring WJ, Mirault M-E, Goldschmidt-Clermont M, Moran L, Tissieres A (1978): Two hybrid plasmids with D. melanogaster DNA sequences complementary to mRNA coding for the major heat shock protein. Cell 14:921-929.

11. Mitchell HK (1966): Phenox oxidases and Drosophila development. J Insect Physiol 12:755-765.

12. Goldschmidt R (1935): Gen und Ausseneigenschaft (Untersuchung an Drosophila) I, II. Zeitschr. f. indukt. Abst. - u. Vererbungslehre 69:38-69, 70-131.

13. Gloor H (1945): Phänokopie einer Letal mutante (cvc) von Drosophila melanogaster. Rev Suisse Zool 51:394-402.

14. Milkman R (1962): Temperature effects on day old Drosophila pupae. J Gen Physiol 45:777-799.

15. Mitchell HK, Lipps LS (1978): Heat shock and phenocopy induction in Drosophila. Cell 15:907-918.

16. Mitchell HK, Lipps LS, Tracy UM (1977): Transcriptional changes in pupal hypoderm in Drosophila melanogaster. Biochem Genet 15:575-587.

17. Moller G, manuscript in preparation.

18. Mitchell HK, Mitchell A (1964): Mass culture and age selection in Drosophila. Drosophila Inf Serv 39:135-137.

19. Fairbanks G, Steck TL, Wallach DFH (1971): Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606-2617.

20. Laemmli U (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

21. Weber K, Osborn M (1969): The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406-4412.

22. Ish-Horowicz D, Holden JJ, Gehring W (1977): Deletions of two heat-activated loci in Drosophila melanogaster and their effects on heat-induced protein synthesis. Cell 12:643-652.

23. Lindsley DE, Poodry CA (1977): A reversible temperature-induced developmental arrest in Drosophila. Develop Biol 56:213-218.

CHAPTER III

GENETIC MAPPING OF THE CODING REGIONS FOR THREE HEAT SHOCK PROTEINS IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

We describe variants of three heat shock proteins of Drosophila melanogaster and the use of these to map the chromosome regions which contain the coding sequences for these proteins. All three map to a region on chromosome 3L which includes only one heat shock puff, that designated as 67B. The results imply that the genes which code for at least three heat shock proteins are included within the 67B region.

INTRODUCTION

The changes in gene expression following heat shock in Drosophila melanogaster provide a useful system for studying the regulation of eukaryotic gene transcription in a group of genes which are coordinately expressed. The heat shock response was first observed as the induction of 9 specific puffs on salivary gland chromosomes following a brief shift to temperatures between 35° and 40° (RITOSSA, 1962; ASHBURNER, 1970). In 1974 TISSIÈRES, MITCHELL and TRACY showed that there were changes in the pattern of protein synthesis in several Drosophila tissues which paralleled the changes in the chromosomal puffing patterns. In particular 7-9 new proteins were synthesized after heat shock and it was hypothesized that these proteins were coded at the sites of the heat shock puffs. This hypothesis was substantiated by the observation that heat shocked cell lines make new mRNA which hybridizes in situ to the heat shock puffs of the salivary gland chromosomes and which directs the synthesis of heat shock proteins in in vitro protein synthesis systems (McKENZIE et al., 1975; SPRADLING, PENMAN and PARDUE, 1975; SPRADLING, PARDUE and PENMAN, 1977; MORAN et al., 1978; MIRAULT et al., 1978).

The question of which heat shock puffs code for which heat shock proteins can be answered using genetic techniques or by in situ hybridization of purified mRNA to polytene chromosomes and in vitro transcription of the same mRNA. Most of the information so far has come from in situ hybridization of purified mRNA to polytene chromosomes and in vitro translation of the same mRNA. The puffs coding for the three larger heat shock proteins, the 84K, 70K, and 68K proteins, have been identified in this way. The identification of the coding regions for the smaller heat shock proteins, the 22K, 23K, 26K and 27K proteins has been

less successful because their messengers are similar in size and are made in smaller quantities. We have identified electrophoretic variants of three of these proteins and mapped their genetic position by linkage to recessive visible markers. All three map to a region of the chromosome which contains only one heat shock puff, the 67B puff.

MATERIALS AND METHODS

Drosophila cultures and stocks: Flies were raised at 25° on standard cornmeal medium (LEWIS, 1960). Both wild-type cultures, Urbana S and Canton S, and the strains with visible markers In(2L+2R)Cy, Cy sp^2/In(2LR)bw^{V1}, ds^{33k} dp b bw^{V1}; In(3LR)DcxF, ru h D/In(3R)Mo, Sb sr sp and ru h th st cu sr e^s ca (rucuca) were obtained from the Drosophila Culture Center (California Institute of Technology, Pasadena). These mutations are described by LINDSLEY and GRELL (1968). DTS-1 was generously supplied by D. T. Suzuki (HOLDEN and SUZUKI, 1973).

Labeling of ovaries with ^{35}S methionine: Adult females between two days and two weeks old were heat shocked for 40 min by submersing vials containing the flies in a 37.5° water bath. Immediately following the heat treatment ovaries were removed and placed in a drop of buffered saline containing 10 µCi/µl ^{35}S Met (~300 Ci/mole) as described by MITCHELL et al. (1977). The ovaries were incubated for 25 min at room temperature (~25°) and then put in cold 10% TCA for at least 30 min. They were washed three times in 95% ETOH, dried, and each pair of ovaries was dissolved in 25-30 µl of sample buffer by heating 10 min in boiling water.

SDS gradient gels: Electrophoresis was carried out as described by TISSIÉRES

et al. (1974) except that the separating gel was a 10-20% exponential gradient gel (VAN BLERKOM and MANES, 1974) and the acrylamide solutions contained 1.33% bisacrylamide. The modified sample buffer contained: 0.01 M Tris base, 0.01 M Na_2H_2 EDTA, 10% sucrose, 1% SDS, 1% pyronine Y. The final pH was 7.75, and 60 mM dithiothreitol was added on the day the buffer was used.

Electrophoresis was carried out at room temperature and using a constant current of 16 mA for a 22 x 12 x 0.1 cm gel. Gels were fixed in 25% isopropyl alcohol, 10% acetic acid and stained and destained according to FAIRBANKS et al. (1971). Kodak 5B-5 X-ray film was used for autoradiography of the dried gels.

Two-dimensional gels: The isoelectric focusing gels were run according to the procedure of O'FARRELL (1975) as modified by AMES and NIKAIDO (1976) and by CHOMYN (1979). The second dimension was run as described above for SDS gradient gels.

RESULTS

Description of electrophoretic variants of three small heat shock proteins

26-28.5K proteins: Each wild-type stock of flies which we have tested has three heat shock proteins which run in the 26-28.5K region in SDS acrylamide gels. The molecular weights of the two slower proteins from this region are different in different stocks. Figure 1 shows the labeled proteins made by adult ovaries from Urbana S (UrS), Canton S (CS), and rucuca strains after heat shock. The UrS stock has heat shock proteins running at about 28.5K, 27.3K and 26K, the Canton S at 28.0K, 27.0K and 26K, and the rucuca at 27.3K, 26.8K and 26K.

For convenience we have assigned letters to each variant in order of

FIGURE 1. Labeled ovary proteins from three different fly stocks showing differences in the 26.5-28.5K heat shock proteins. Lanes 1, 2, and 3 show ^{35}S methionine labeled proteins synthesized by UrS, CS and rucuca ovaries, respectively, after 40 minutes at 37.5°. Positions of the major heat shock proteins and the variants a, b, c, d, and e are indicated. Lanes 4, 5, and 6 show ^{35}S labeled proteins made by UrS, CS, and rucuca ovaries, respectively, that have not been heat shocked.

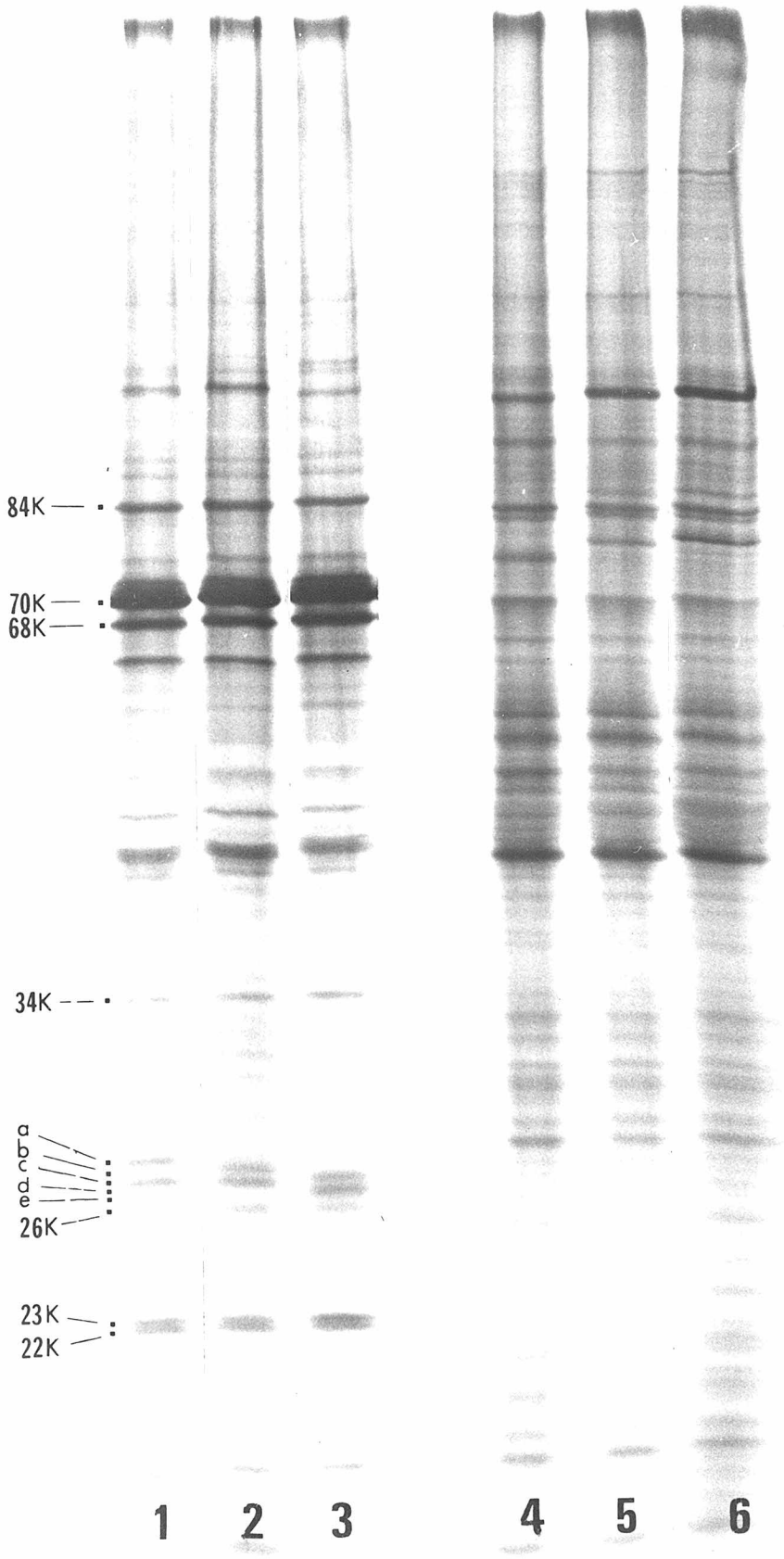

84K
70K
68K
34K
a
b
c
d
e
26K
23K
22K
1
2
3
4
5
6

TABLE 1

Electrophoretic variants

	Stock of origin	Molecular weight range
a	UrS	28.1-28.5K
b	CS	27.5-28.0K
c	UrS, *rucuca*	27.1-27.5K
d	CS	26.8-27.2K
e	*rucuca*	26.5-26.8K

FIGURE 2. Autoradiogram of heat shock proteins from CS and UrS flies run on a two-dimensional gel. Autoradiogram of a two-dimensional gel. ^{35}S methionine labeled heat shock proteins from CS flies were run on one half of the gel and UrS labeled heat shock proteins were run on the other half.

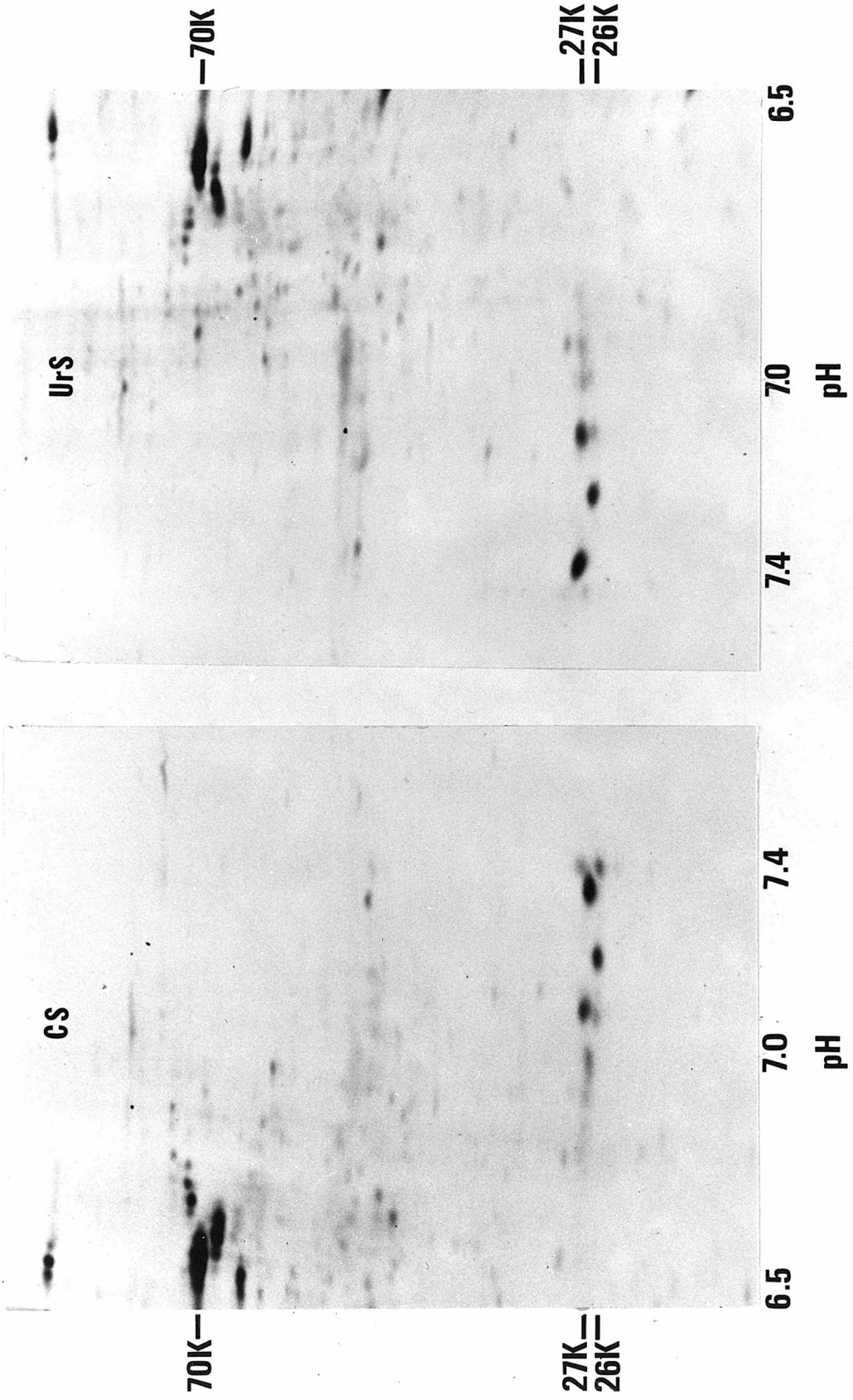
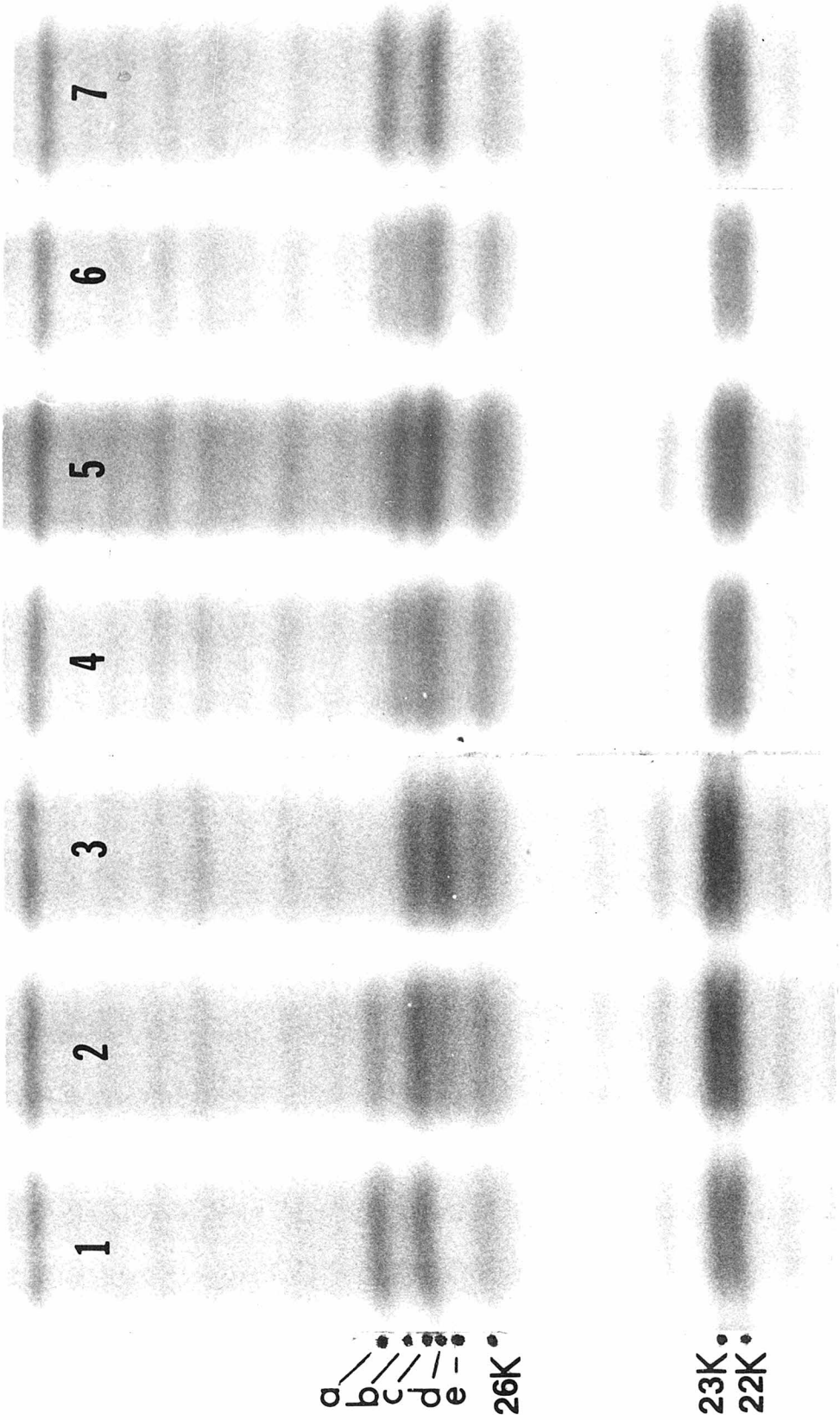

CS
UrS
70K
27K
26K
6.5
7.0
7.4
pH

FIGURE 3. Labeled heat shock proteins from flies showing a normal and a fast running 23K protein. Heat shock ovary proteins from progeny and parents in the cross DTS1/TM2 ♀ x rucuca ♂ were run on SDS 10-20% exp gradient gels. Flies were heat shocked and ovaries labeled as described in Materials and Methods. Lane (1) DTS1/rucuca; (2) TM2/rucuca; (3) DTS1/TM2; (4) rucuca.

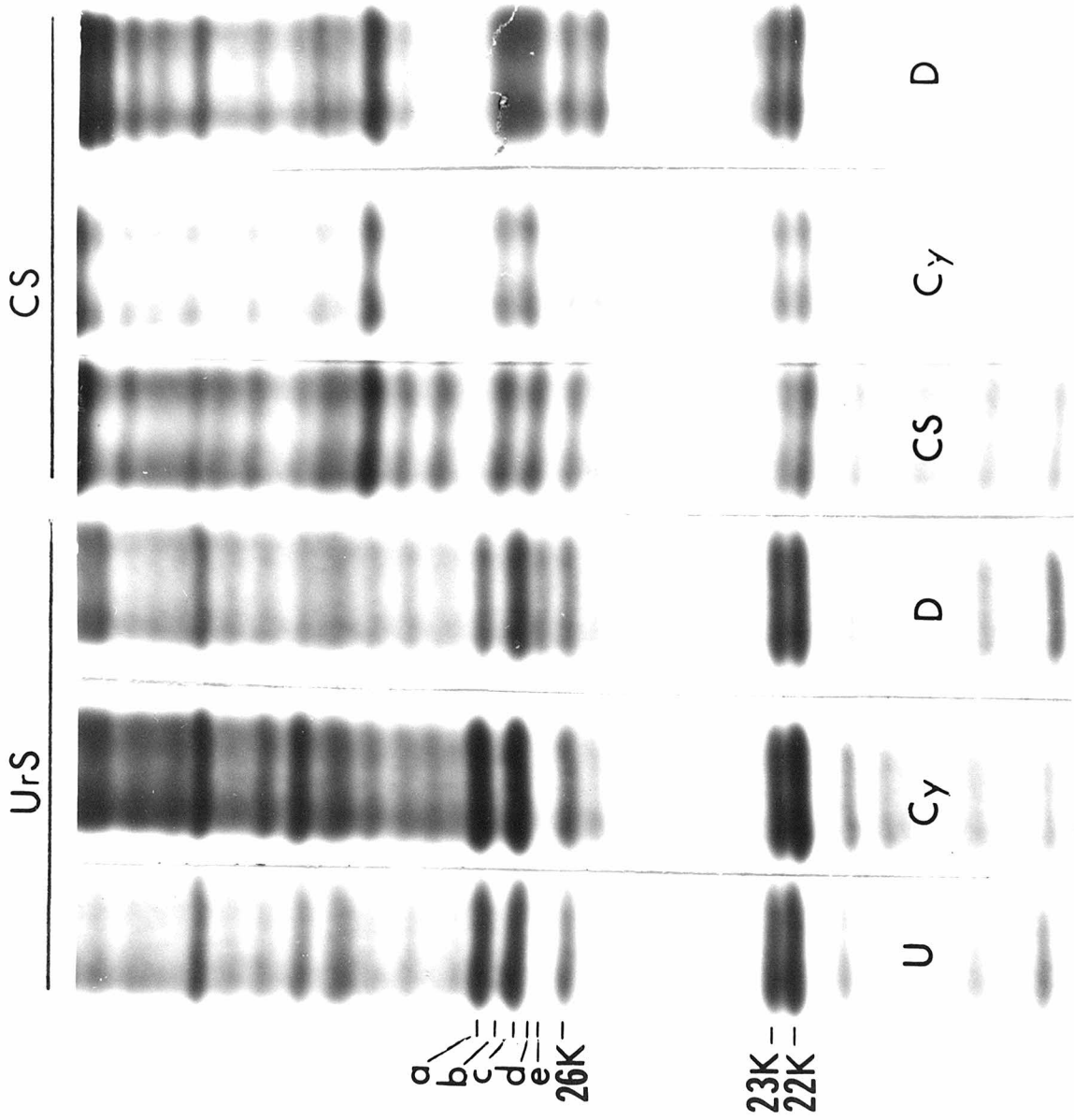

1
2
3
4
5
6
7
a
b
c
d
e
26K
23K
22K

its apparent molecular weight as listed in Table 1. When these proteins are run on two-dimensional gels of the O'Farrell (1975) type, three major components are again resolved. Figure 2 shows heat shock proteins from Urbana S and Canton S flies. Three proteins in the 26-28.5K region are seen, each with a distinct isoelectric point; however, some resolution in the SDS dimension is lost so that differences between the 27-28.5K proteins in the CS and UrS flies are not resolved.

Electrophoretic variation in the 23K protein: We found a variant of the 23K protein in a stock of DTS1/TM2 which was received from Suzuki's lab in Vancouver. The variant ran faster than the normal 23K protein, at approximately 22.6K, and was derived from both the DTS1 chromosome and the TM2 chromosome as shown in Figure 3. It will be shown later that the 22.6K protein is not linked to the DTS1 mutation which maps at about 78 map units on the right arm of the third chromosome (HOLDEN and SUZUKI, 1973).

Chromosomal localization of the 26.5-28.5K heat shock proteins using semidominant heterozygote heat shock phenotypes: Heat shock puffs occur on both the second and third chromosomes. In order to determine which chromosome contains the coding region for the 26.5-28.5K heat shock proteins we crossed UrS females and CS females to males carrying dominant markers Curly (Cy) on the second chromosome and Dichaete (D) on the third chromosome. From the F_2 progeny we picked females which carried all UrS or all CS chromosomes except for one marker chromosome, either a second chromosome recognized by Cy, or a third chromosome recognized by D. Labeled ovary proteins from individual flies of each phenotype were run on SDS gels. As shown in Figure 4 the 27K heat shock proteins from flies heterozygous for the marker second chromosome are identical to either CS or UrS heat shock proteins. The flies heterozygous for

FIGURE 4. Small heat shock proteins from progeny of the crosses UrS ♀ x Cy/UrS, D/UrS ♂ and CS ♀ x Cy/CS; D/CS ♂. Flies were heat shocked and ovaries labeled with ^{35}S methionine and run on SDS acrylamide gels as described in Materials and Methods. The positions of the different 26.5-28.5K heat shock proteins are indicated by a, b, c, d, and e. Lanes marked U or CS indicate homozygous UrS or CS, respectively. Lanes marked Cy or D indicate flies with one second chromosome marked with Cy or one third chromosome marked with D.


UrS
CS
U
Cy
D
CS
Cy
D
a
b
c
d
e
26K
23K
22K

third chromosomes marked with D show very different patterns for the 27K proteins, indicating that the differences in the 27K bands are coded on the third chromosome. Densitometer tracings of these gels (Figure 5) show that the UrS band a decreases by about 50% in intensity and a new band appears at the position of the e protein. The intensity of the c band is unchanged. The simplest explanation of these results is that the marker chromosome codes for bands c and e. If this is the case then the amount of gene product is proportional to the gene dose and there is twice as much c protein in the heterozygote as either a or e protein because the c protein gene is present in both genotypes on third chromosomes while the a and e genes are present only once on UrS and D third chromosomes, respectively. This could not be tested further because homozygous D is lethal. This also explains the appearance of a broad band in the CS/D hybrid which goes from the b to e position and could include gene products b and d from CS and c and e from D. In other gels we have been able to resolve b from c, d and e but not d and e from c in the same sample. In crosses between UrS and CS we were able to resolve a from b but not c from d. In order to see whether these bands could be resolved in our gel system we ran a gel containing each stock type separately and also mixtures of UrS and CS, UrS and rucuca, and CS and rucuca in which the samples were prepared separately and mixed immediately before running the gel. Shown in Figure 6 are Urbana S, Canton S, and rucuca 26-28K proteins and the possible mixtures. As in the heterozygotes the mixture between UrS and rucuca can be resolved into a, c, and e. The mixtures between UrS and CS and between CS and rucuca do not show c, d, and e as separate bands. It is still possible to use the CS/rucuca heterozygote to map the position of bands b, c, and d since for mapping, one is comparing CS/rucuca hybrid to the rucuca type and it is easy to recognize the appearance of bands b and d, and the decrease in intensity in the c and e regions.

FIGURE 5. Densitometer tracings of the autoradiogram shown in Figure 4. The letters a, b, c, d, and e indicate the positions of the different 26.5K to 28.5K proteins as defined in Table 1. U = Urbana S; C, Canton S.

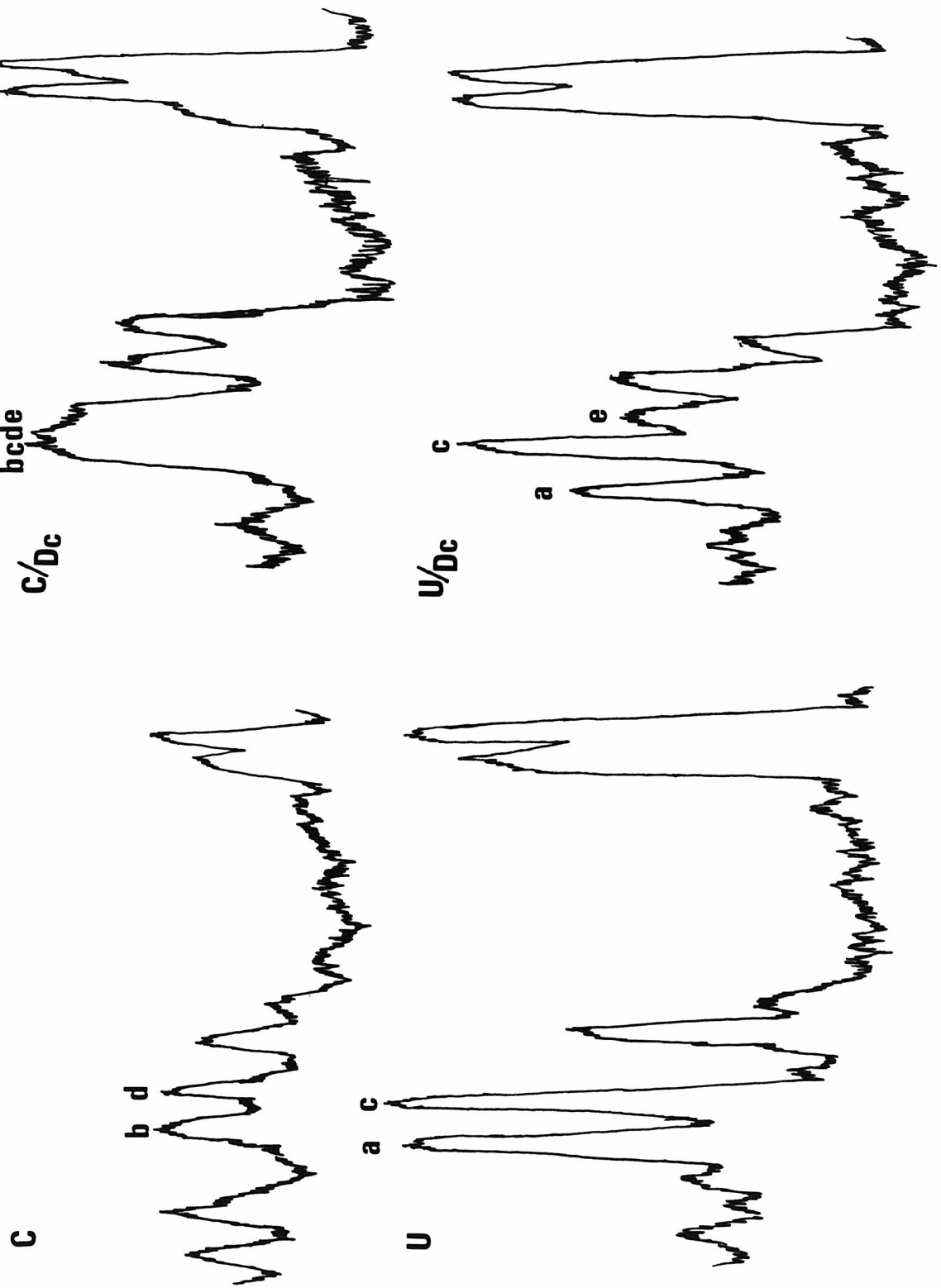
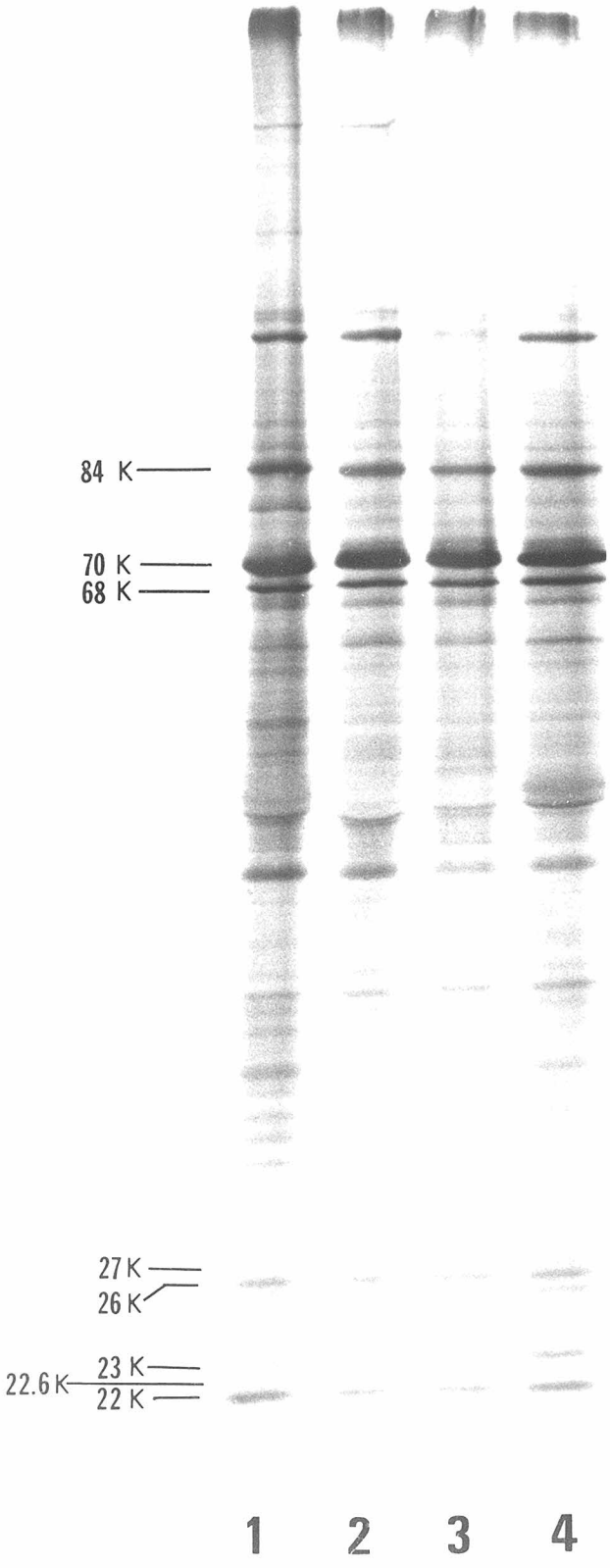

C
b d
C/Dc
bcde
U
a c
U/Dc
a
c
e

FIGURE 6. The 26-28.5K heat shock proteins from UrS, CS and rucuca flies and different mixtures containing protein from two of these strains. The flies were heat shocked and ovaries labeled with ^{35}S Met as described previously. Immediately before running on gels, samples 2, 4 and 6 were made by mixing equal amounts of samples 1 and 3, 3 and 5, and 5 and 7. (1) Urbana S; (2) UrS + rucuca; (3) rucuca; (4) rucuca + CS; (5) CS; (6) CS + UrS; (7) UrS. The positions of the 26.5-28.5K proteins are indicated by letters a, b, c, d, and e defined in Table 1.


84 K
70 K
68 K
27 K
26 K
22.6 K
23 K
22 K
1
2
3
4

Mapping of the coding regions for the 26.5-28.5K heat shock proteins: We used the rucuca chromosome with eight visible markers to map the position on the third chromosome of the genes coding for the electrophoretic variants. Both UrS and CS females were crossed to rucuca males as shown in Table 2. Heterozygous females were picked from each cross and backcrossed to rucuca males. Recombinant progeny were picked which showed different combinations of the recessive visible markers. The heat shock phenotypes of flies showing different combinations of visible markers are tabulated in Table 2. Where the visible recessive markers include the region coding for 26.5-28.5K proteins we see the rucuca heat shock phenotype of bands c and e. Where the recessive markers do not include the region coding for the 26.5-28.5K proteins we see the heterozygote pattern. These experiments show that a, b, c, and d and e all map between thread and hairy. There are two heat shock puffs between hairy and thread, at 67B and at 70A, so more recombinants with phenotypes of ru, h, th, and st were examined to determine the approximate coding positions of the variants. Table 3 shows that genes for all four polypeptides are linked to hairy at about 2.4 map units to the right. Hairy is located between 66D2 and 66E1 and thread is located between 72A2 and 72EF (LINDSLEY and GRELL, 1968). Assuming an approximately linear relationship between map units and salivary chromosome bands (LEFEVRE, 1971) we conclude that the only heat shock puff in the region which codes for these proteins is 67B.

Mapping of the coding region for the 23K protein: The mapping of the coding region for the 23K protein was more straightforward since there is only one protein and one fast running allele. The possibility that this protein was coded on the second chromosome was eliminated by backcrossing TM2/rucuca females

TABLE 2

Localization of the 26.5-28.5K protein genes on the third chromosome

ru	h	th st cu	sr	e^s	ca*
0.0	26.5	43.2 44 50.0	62.0	70.7	100.7

63BC 64F 67B 70A 87A-C 93D 95D

UrS/rucuca ♀ x rucuca ♂

F_1 visible phenotype	Heat shock phenotype rucuca	Heat shock phenotype UrS/rucuca
$\underline{e^s}$ ca	0	1
sr $\underline{e^s}$ ca	0	1
cu sr $\underline{e^s}$ ca	0	2
ru h th st	2	0
h th st	1	0
ru h	3	2
th st	0	4

CS/rucuca ♀ x rucuca ♂

F_1 visible phenotype	Heat shock phenotype rucuca	Heat shock phenotype CS/rucuca
sr $\underline{e^s}$ ca	0	5
ru h th st cu	5	0
th st cu sr	0	3
h th st	1	0
ru h	1	1

*Above the line are the third chromosome visible markers seen in the rucuca stock. The position of these markers on the crossover map is indicated. For description see LINDSLEY and GRELL (1968). Below the line are the approximate positions of the heat shock puffs.

TABLE 3

Mapping of the coding regions for the 26.5-28.5K proteins

UrS/rucuca ♀ x rucuca ♂

VISIBLE PHENOTYPE	No.	HEAT SHOCK PHENOTYPE UrS/rucuca	rucuca	%
ru h	53	8	45	15
th st	45	39	6	13

distance from hairy = 16.7 mu x 14% = 2.4 mu (1.4-4.5 mu)*

CS/rucuca ♀ x rucuca ♂

VISIBLE PHENOTYPE	No.	HEAT SHOCK PHENOTYPE CS/rucuca	rucuca	%
ru h	26	3	23	12
th st	24	20	4	17

distance from hairy = 16.7 x 14% = 2.4 mu (0.7-5.3)*

*Distance in parentheses indicate the limits of the 99% confidence interval for the distance from hairy.

to rucuca males and picking TM2/rucuca progeny. Of the six progeny tested all showed both 23K and 22.6K bands which indicates that the coding region for these two proteins is on the third chromosome. The region of the chromosome which codes for these proteins was mapped with respect to recessive markers on the rucuca third chromosome in the same way as for the 26.5-28.5K proteins. Table 4 shows that the coding region for these proteins is also located between h and th at approximately 2.6 map units to the right of hairy. Again the only heat shock puff in this region is 67B.

DISCUSSION

We have shown that the genes in the 67B region are responsible for the appearance of electrophoretic variants of three heat shock proteins. The data can be interpreted in several ways:

1) The structural genes for the 23K protein and two proteins called 27K proteins are included in the 67B region. If there are only three genes coding for these proteins in each 67B region, then 23K and 22.6K are alleles, the 27a and 27e are alleles and 27b and 27d are different genes, one of which is allelic to 27a and the other to 27c. On the other hand, all of the 27K proteins may be members of a tandemly repeated gene family and may be pseudoalleles of one another.

2) The electrophoretic variants may result from post-translational modification of a protein which may or may not be coded in 67B, but the modifying enzymes are coded in 67B. This is not the case for the 23K protein and at least one 27K protein since the 23K and at least one 27K protein are made in an in vitro protein synthesizing system from heat shock mRNA (MORAN et al., 1978; McKENZIE and MESELSON, 1977). It is possible that one of the 27K proteins

TABLE 4

Mapping of the coding regions for the 23K protein

Visible markers	Heat shock phenotypes		
	rucuca	DTS1/rucuca	%
e^s ca		1	
th st sr cu e^s ca	1	4	20
ru h	23	4	15
ru e^s ca		1	

distance from hairy - 5/32 (16.7 mu) = 2.6 mu (0.5-6.7)*

*Distance in parentheses indicates the limits of the 99% confidence interval for the distance from hairy.

is a modified form of the other. If this is the case, the modification must occur very rapidly since relative amounts of the labeled proteins do not differ in samples labeled five minutes and those labeled for 25 min (PETERSEN, unpublished).

3) Finally there is the possibility that the message coding for these proteins originates somewhere else in the chromosome, but that enzymes which process the message are coded at 67B. There is no evidence favoring this hypothesis, but it cannot be ruled out on the basis of our data.

We favor the first interpretation of the data which is that the coding regions for these proteins are included in the 67B region. In agreement with this interpretation, a genomic DNA clone from the 67B region has been identified and has been shown to contain sequences coding for the 23K protein and the 26K protein (Elizabeth Craig, personal communication). The tryptic fingerprints of the 26K protein show some similarities to at least one 27K protein (MIRAULT et al., 1977). This supports the idea that the 67B region may code for a group of tandemly repeated similar genes.

The earlier work on chromosome puffs and protein synthesis in salivary glands of Drosophila melanogaster (TISSIÈRES et al., 1974) encouraged the tacit assumption that each puff induced by heat shock yields a single mRNA and a single polypeptide product. Subsequent work has not abrogated this assumption but evidence has been presented that two different puff regions code for multiple copies of messenger for the same protein (ISH-HOROWICZ et al., 1977; SCHEDL et al., 1978; LIS et al., 1978). Now, as we have shown, the coding regions for three different heat shock proteins map to a segment of chromosome which contains only one heat shock puff (67B). This puff includes 13 bands by the Bridges' map (LINDSLEY and GRELL, 1968) and thus a segment of DNA on the order of 200 kilobase pairs. This is more than ample coding capacity for the three proteins

in question and many more. As to the question of how much of this coding capacity is involved in transcriptional activity in the heat shock-puff state, only limited information is available at present. Data from in situ hybridization (McKENZIE et al., 1977; MORAN et al., 1978; MIRAULT et al., 1977) show that a mixed message RNA fraction which can be translated in vitro to yield 28, 27, 26, 23 and 22K proteins hybridizes mainly to the 67B chromosome region. This can mean that all of the messages are derived from this region but it can also mean that only one originates in this particular segment. Our observations from autoradiography of transcriptional activity within the 67B region are that at least two and more likely three bands are active in the heat shock situation. Details will be presented elsewhere. It seems clear that additional studies coupled with appropriate clone selection will be needed to evaluate further the structure-function relations of the 67B region.

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LITERATURE CITED

AMES, G. F.-L. and K. NIKAIDO, 1976 Two-dimensional gel electrophoresis of membrane proteins. Biochemistry **15:** 616-623.

ASHBURNER, M., 1970 Patterns of puffing activity in the salivary gland chromosomes of Drosophila. V. Responses to environmental treatments. Chromosoma (Berl.) **31:** 356-376.

CHOMYN, A., 1979 Thesis, California Institute of Technology, Pasadena, California.

FAIRBANKS, G., T. L. STECK and D. F. H. WALLACH, 1971 Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemstry **10:** 2606-2617.

HENIKOFF, S. and M. MESELSON, 1977 Transcription at two heat shock loci in Drosophila. Cell **12:** 441-451.

HOLDEN, J. J. and D. T. SUZUKI, 1973 Temperature-sensitive mutations in Drosophila melanogaster. XII. Genetic and developmental effects of dominant lethals on chromosome 3. Genetics **73:** 445-458.

ISH-HOROWICZ, D., J. J. HOLDEN and W. J. GEHRING, 1977 Deletions of two heat-activated loci in D. melanogaster and their effects on heat-induced protein synthesis. Cell **12:** 643-652.

LEFEVRE, G., 1971 Salivary chromosome bands and the frequency of crossing over in Drosophila melanogaster. Genetics **67:** 497-513.

LEWIS, E. B., 1960 A new standard food medium. DIS **34:** 117.

LEWIS, M., P. J. HELMSING and M. ASHBURNER, 1975 Parallel changes in puffing activity and patterns of protein synthesis in salivary glands of Drosophila. Proc. Natl. Acad. Sci. U.S. **72:** 3604-3608.

LINDSLEY, D. L. and E. H. GRELL, 1968 Genetic Variations of Drosophila melanogaster. Carnegie Inst. Wash. Publ. **627.**

LIS, J. T., L. PESLIDGE and D. S. HOGNESS, 1978 A novel arrangement of tandemly repeated genes at a major heat shock site of D. melanogaster. Cell **14:** 901-920.

McKENZIE, S. L., S. HENIKOFF and M. MESELSON, 1975 Localization of RNA from heat induced polysomes at puff sites in D. melanogaster. Proc. Natl. Acad. Sci. U.S. **72:** 1117-1121.

McKENZIE, S. L. and M. MESELSON, 1977 Translation in vitro of Drosophila heat-shock messages. J. Mol. Biol. **117:** 279-283.

MIRAULT, M., M. GOLDSCHMIDT-CLERMONT, L. MORAN, A. P. ARRIGO and A. TISSIÉRES, 1977 The effect of heat shock on gene expression in D. melanogaster. Cold Spring Harbor Symp. on Quant. Biol. **42:** 819-827.

MITCHELL, H. K. and L. S. LIPPS, 1975 Rapidly labeled proteins on the salivary gland chromosomes of D. melanogaster. Biochem. Genet. **13:** 585-602.

MITCHELL, H. K., L. S. LIPPS and U. M. TRACY, 1977 Transcriptional changes in pupal hypoderm in Drosophila melanogaster. Biochem. Genet. **15:** 575-587.

MORAN, L., M.-E. MIRAULT, A. P. ARRIGO, M. GOLDSCHMIDT-CLERMONT and A. TISSIÉRES, 1978 Heat shock in D. melanogaster induces synthesis of new mRNAs and proteins. Transact. of Royal Soc. **283:** 391-406.

O'FARRELL, P. H., 1975 High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:** 4007-4021.

RITOSSA, F., 1962 A new puffing pattern induced by temperature shock and DNA in Drosophila. Experientia (Basel) **18:** 571-573.

SCHEDL, P., S. ARTAVANIS-TSAKONAS, R. STEWARD, W. J. GEHRING, M.-E. MIRAULT, M. GOLDSCHMIDT-CLERMONT, L. MORAN and A. TISSIÉRES, 1978 Two hybrid plasmids with Drosophila melanogaster DNA sequences complementary to mRNA coding for the major heat shock protein. Cell **14**, 921-929.

SPRADLING, A., M. L. PARDUE and S. PENMAN, 1977 Messenger RNA in heat-shocked Drosophila cells. J. Mol. Biol. **109**: 559-587.

SPRADLING, A., S. PENMAN and M. L. PARDUE, 1975 Analysis of Drosophila mRNA by in situ hybridization: Sequences transcribed in normal and heat shocked cultured cells. Cell **4**: 395-404.

TISSIÉRES, A., H. K. MITCHELL and U. M. TRACY, 1974 Protein synthesis in salivary glands of Drosophila melanogaster. Relation to chromosome puffs. J. Mol. Biol. **84**: 389-398.

VAN BLERKOM, J. and C. MANES, 1974 Development of preimplantation rabbit embryos in vivo and in vitro. 2. Comparison of qualitative aspects of protein synthesis. Develop. Biol. **40**: 40-51.

CHAPTER IV

Specific Protection from Phenocopy Induction by Heat Shock

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Summary

Mild heat treatments applied to whole animals or cell cultures of Drosophila melanogaster prior to lethal heat shocks result in both survival and protection against phenocopy induction. From an examination of these heat shock effects on transcriptional and translational activities in tissues and cells it appears that the protective action of pretreatment is due to sequestering of mRNAs in a masked form as RNPs. Heat shock proteins are evidently involved in the masking process either directly or indirectly.

Introduction

We described recently (Mitchell and Lipps, 1978) a series of phenocopies that are induced in Drosophila melanogaster by subjecting pupae to heat shocks at specific stages of development. The evidence presented supports the view that each phenocopy is derived from a single unique event which involves the production and dispensation of a particular messenger RNA. In relation to the work described here, the salient observations made earlier in this laboratory (Mitchell and Lipps, 1978; Chomyn, Moller and Mitchell, 1979) concern the facts that heat shocks turn off both transcriptional and translational activities for a time and then translation resumes prior to reactivation of transcription. These various observations have now been extended in the format presented many years ago by Milkman and collaborators (Milkman, 1962, 1966). These investigators demonstrated that if Drosophila pupae (24 hr) were subjected to a mild heat shock prior to a shock that would be lethal alone, many animals would survive. Furthermore it was shown that a pretreatment of this kind would prevent the induction of a phenocopy of the mutant crossveinless.

The protection effects observed by Milkman and collaborators, in conjunction with the apparent fact that protein synthesis resumes after heat shock but well before there is a recovery of RNA synthesis (Mitchell and Lipps, 1978) suggested to us that the heat shock pretreatment phenomenon may involve an accumulation of messages (mRNAs) in a masked form. The evidence presented here provides additional support for such a conclusion.

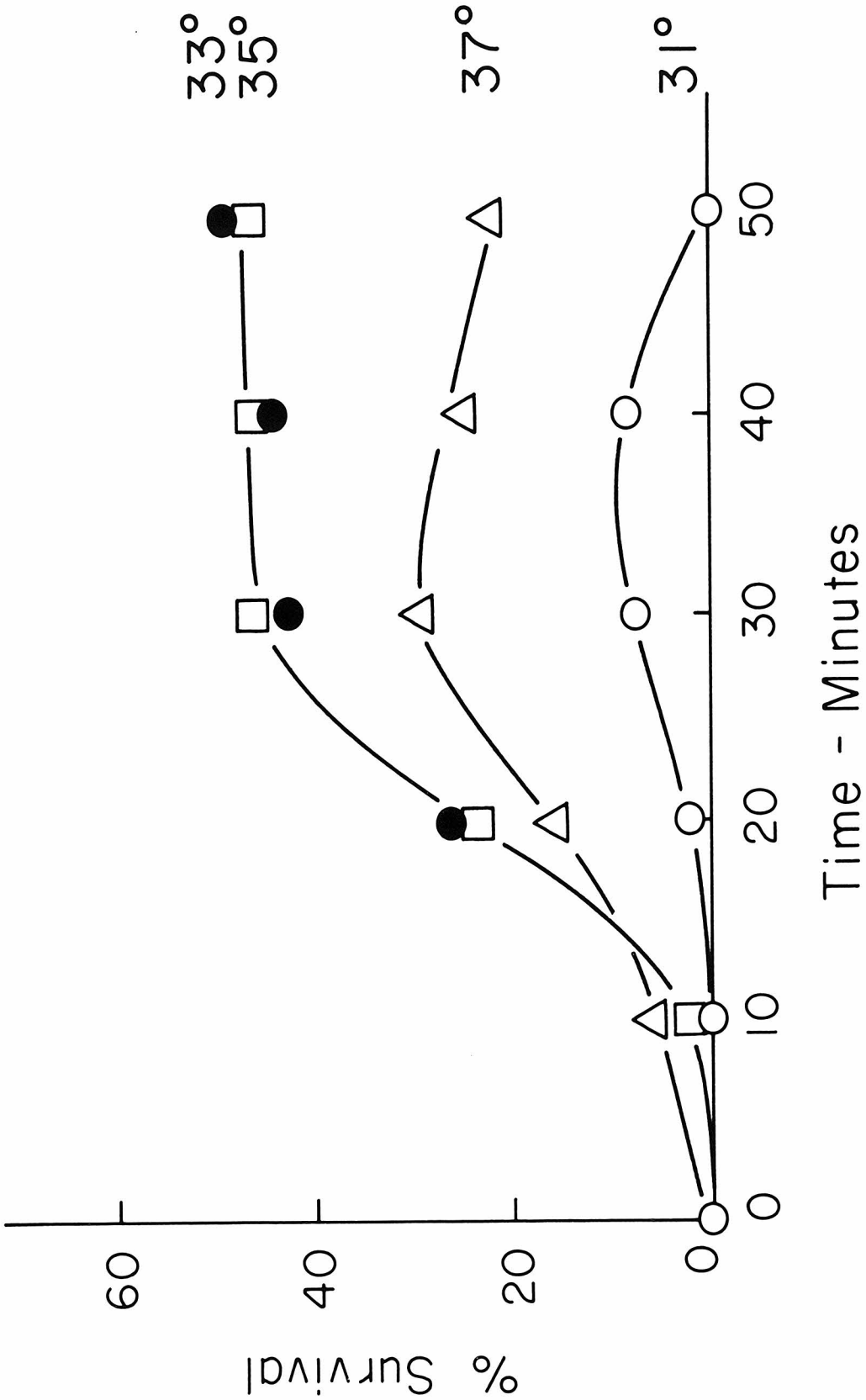
Results

Protection for Survival

As shown in Figure 1, third instar larvae are all killed by a heat shock of 40.5°C

Figure 1. Survival of Larvae Following Heat Shocks

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


33°
35°
37°
31°
% Survival
0
20
40
60
0
10
20
30
40
50
Time - Minutes

for a period of 20 min. However, if the animals are pretreated in the temperature range of 31°C to 37°C and then subjected to the lethal temperature, as many as 50% of the animals survive and develop normally. Some protection is evidenced at temperatures as low as 28°C and as high as 39°C.

The specific conditions for lethality and protection shown for third instar larvae (Figure 1) do not apply to animals at all ages and stages of development but the survival-protection effect does obtain at all stages tested and to cell cultures. Some specific examples are shown in Table 1. These data show the generality of the pretreatment effect down to the cell level and also the relatively greater resistance of pupae (pharate adults) to heat shock. The latter was defined more extensively in connection with earlier work on phenocopies (Mitchell, 1966).

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

Specific Protection by Heat Shock

In an earlier paper (Mitchell and Lipps, 1978) we described a series of stage-specific phenocopies produced by heat shock of pupae in the 30 to 50 hr range. Here we have made use of this series to answer the question of whether pretreatments are effective on a specific basis as well as a general one. As shown in Figure 2 a pretreatment does indeed prevent the production of the specific phenocopy of the mutant hook since the normal scultellar bristles shown in part A were present on all (17) of the animals treated for 40 min at 35°C followed by 30 min at 41.3°C,

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

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

[a]The cell culture line was derived from the Ore-R stock as described by Petersen et al. (1977). Data are given as percent of normal growth rate.

[b]Survival to adult flies.

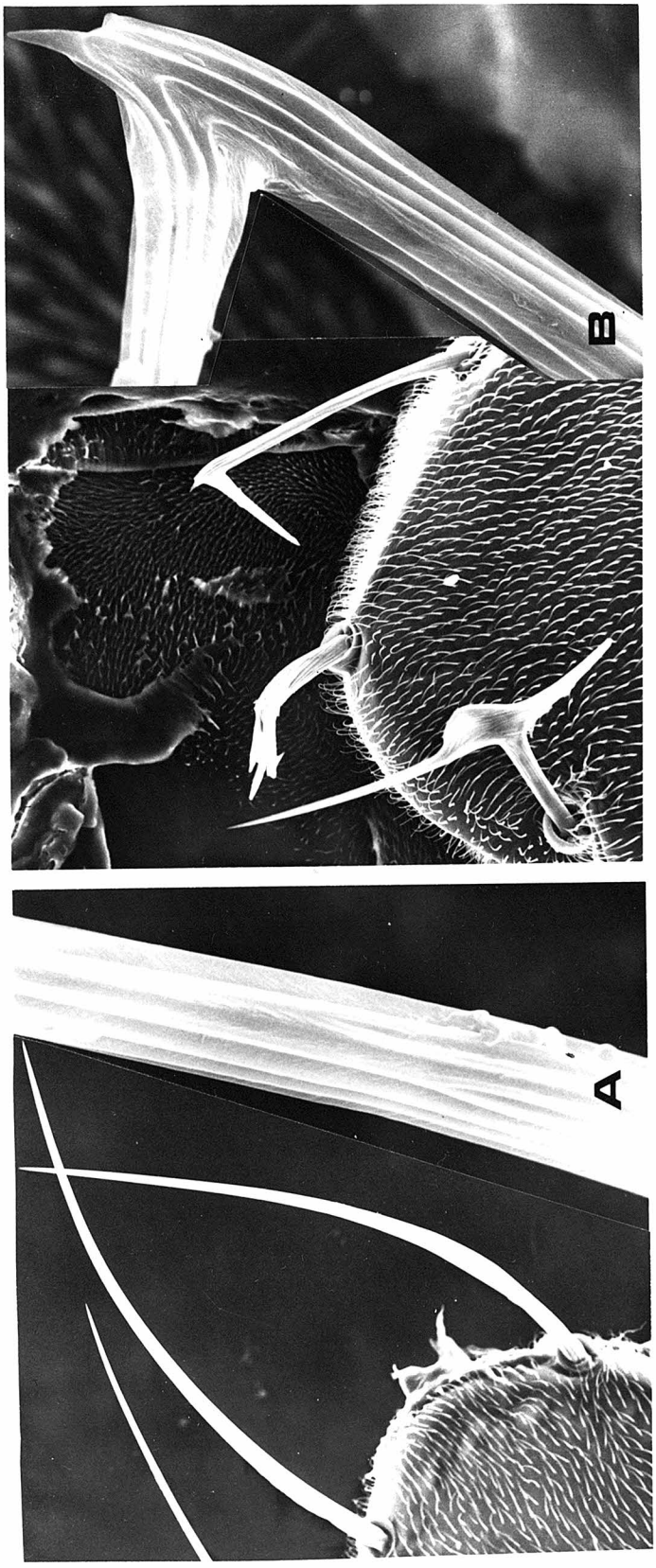
Figure 2. Prevention of Phenocopy Production by a Mild Heat Shock

Pupae synchronized in developmental time to 36 $\pm$ 1 hr after puparium formation were:

A. Treated at 35°C for 40 min and then at 41.3°C for 30 min.

B. Treated at 41.3°C for 30 min.

All animals were kept at 25°C before and after the heat treatments. The photographs (scanning electron microscope) show the scutellum and bristles from an adult fly and a higher magnification of one bristle portion in each case. All adult flies from the pretreated (A) group (17) had normal bristles and all (21) adult flies from the (B) group had abnormal scutellar bristles at least as extreme as those shown.

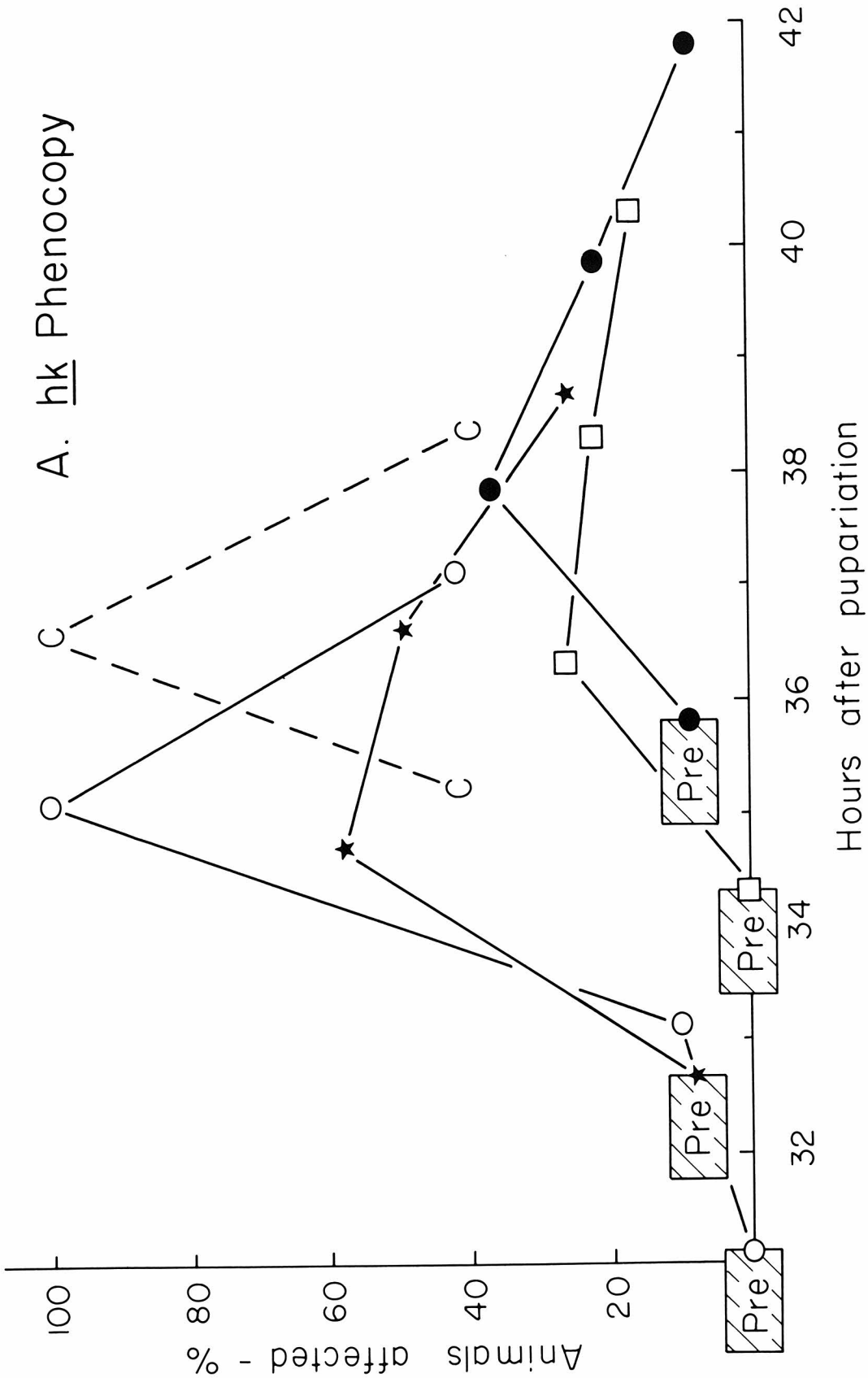

B
A

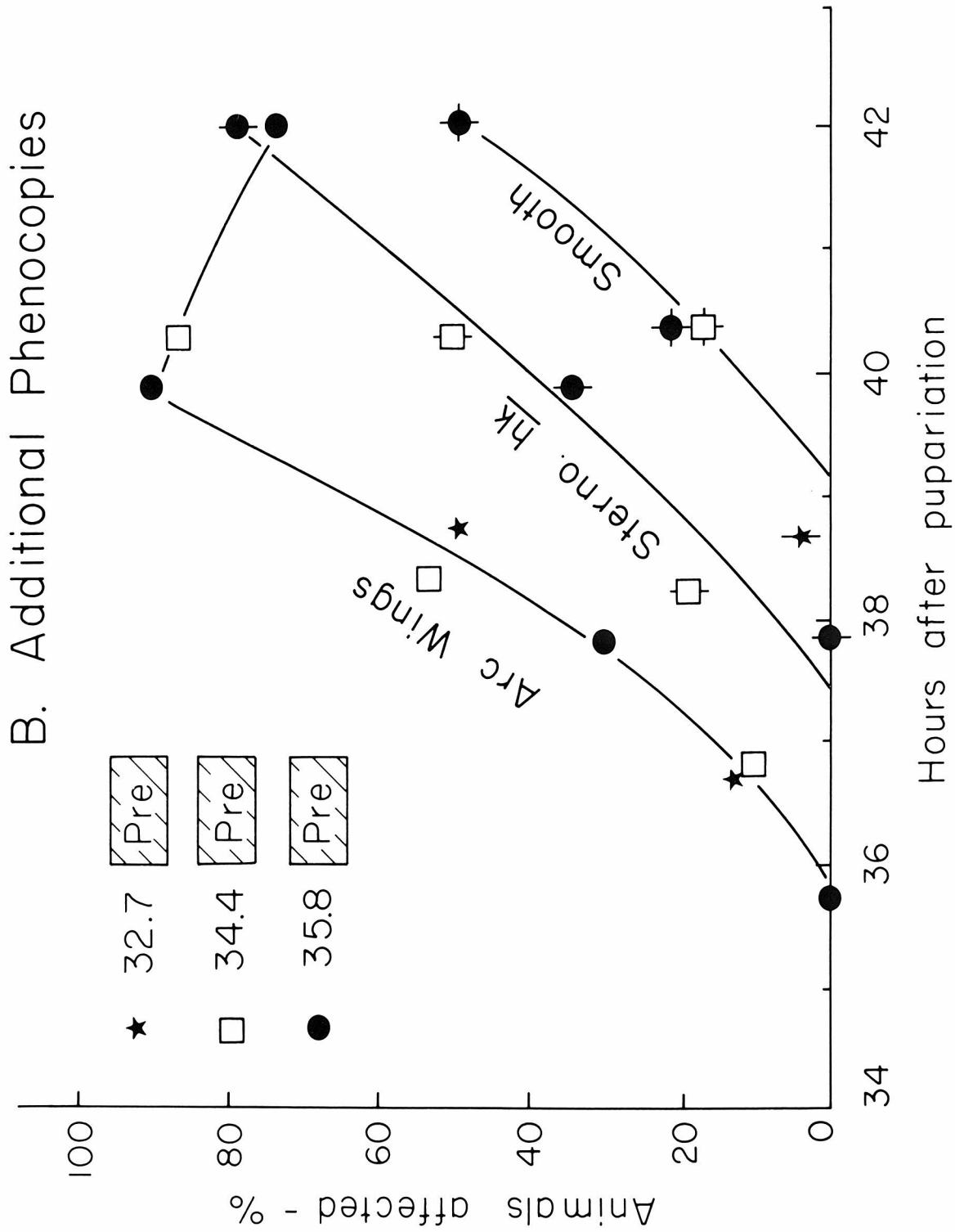
while the hook types shown in part B appeared in all (21) of the flies from the single treatment at 41.3°C for 30 min. This effect is as decisive and dramatic as that shown by the effect of pretreatment on survival (Figure 1) and it could be due to the same general phenomenon. However, a high degree of specificity is shown in this case as demonstrated by the data summarized in Figure 3, parts A and B. In part A it is shown that a pretreatment about 4 hr before the critical period for production of the hook phenocopy (Mitchell and Lipps, 1978) did not affect the subsequent induction of this particular phenocopy. Actually the critical period came a little sooner than in the control as might be expected from the elevation of the temperature during the pretreatment. The second series beginning with a pretreatment around 32 hr (1 to 2 hr before the critical period) resulted in a reduction of the frequency of hook phenocopies. In contrast the pretreatments given at 34 hr and at 35.5 hr (both within the hook sensitive period) gave essentially total protection when the high temperature shock was given immediately after the pretreatment. It is important to note that in both of these latter cases a significant number of phenocopies did appear when the second shock was delayed for an hour after the pretreatment. This shows that the protective agent specific for hook is labile since all pretreated animals showed a much enhanced survival. The data shown in Figure 3B provide additional and essential support to the foregoing conclusions. These results are from the same animals scored for the hook phenocopy shown in part A. They demonstrate that the phenocopies sternopleural hook, arc wings and smooth bristles appeared on schedule regardless of when the pretreatment was given. This supports the contention of independent events giving rise to independent phenocopies as stated earlier (Mitchell and Lipps, 1978).

Figure 3. Specific Effects of Pretreatment on Phenocopy Production

A. The hook phenocopy: the shaded blocks labeled "Pre" indicate the developmental time at which a pretreatment of 34°C for 40 min was given to a synchronous group of 160 animals. In each case 40 pupae were heat shocked immediately after the pretreatment. The remainder were kept at 25°C and 40 pupae were heat shocked subsequently at about 2, 4, and 8 hr after the pretreatment. The pupae were then kept at 25°C until eclosion and the adult flies were scored for the hook phenotype. The dashed line labeled C shows three points for a control set which was not pretreated at 34°C. The entire experiment was done with the same synchronous collection of pupae (760 animals).

B. The same animals scored for hook frequency in part A were scored for the phenocopies arc wing, sternopleural hook and smooth bristles. The symbols used refer to the time of pretreatment. The points for sternopleural hook have a vertical line through the symbols and the points for smooth bristles have both a vertical and a horizontal line.


A. hk Phenocopy
Animals affected - %
100
80
60
40
20
Hours after pupariation
32
34
36
38
40
42
Pre
Pre
Pre
Pre
C
C
C
O
O
O


B. Additional Phenocopies
Animals affected - %
Hours after pupariation
Arc Wings
Sterno. hk
Smooth
Pre
Pre
Pre
32.7
34.4
35.8
0
20
40
60
80
100
34
36
38
40
42

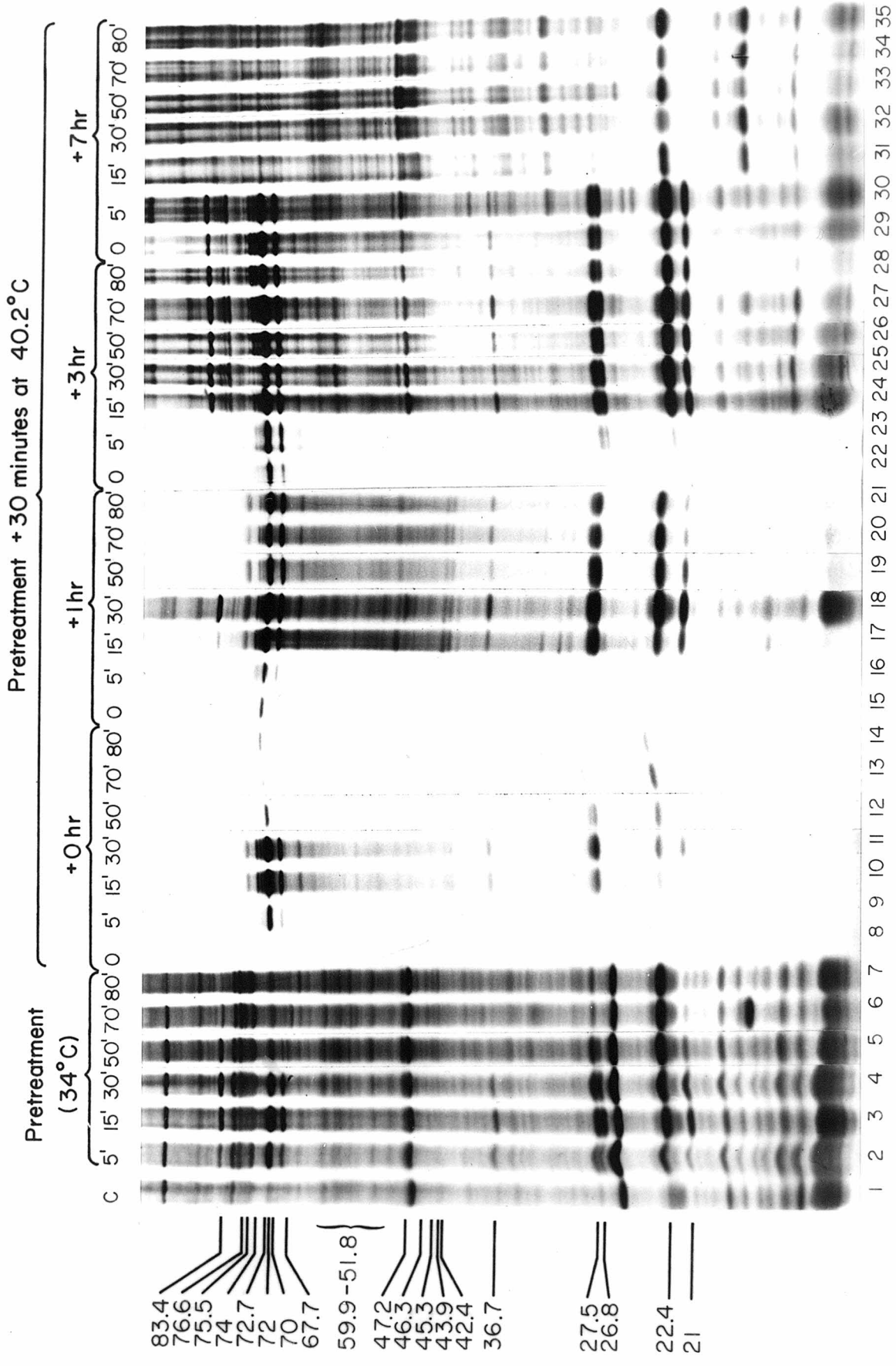
Protection and Protein Synthesis

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

The remaining four sets of samples (tracks 8-35) in Figure 4 show the effects of a second heat shock (40.2°C for 30 min) on a series of samples pretreated as in the series illustrated in tracks 1 to 7. All samples were given the higher temperature shock immediately after the pretreatment but then they were kept at 25°C for 0 hr (tracks 8-14), 1 hr (tracks 15-21), 3 hr (tracks 22-28) and 7 hr (tracks 29-35) before dissection and labeling. Thus these sets of samples show the immediate effects of the high temperature shock on translational capacities and the effects of pretreatments on the recovery of translational capacities. First, in the 0 hr series there is a dramatic reduction in all protein synthesis but protection is evident in the 5 to 50 min range with both normal and heat shock proteins showing strongly at 15 and 30 min (tracks 10 and 11). However, it is

Figure 4. Effects of Pre-Heat Shocks on Protein Synthesis in Salivary Glands

Each sample of the 35 shown (numbers at the bottom) was prepared for electrophoresis on a gradient SDS gel from three pairs of salivary glands dissected from late third instar larvae. The glands were labeled for 20 min at 25°C immediately after the larvae had been subjected to the various heat treatments indicated. The numbers at the left refer to the molecular weights (x 10^3) of the more than 20 heat shock proteins observed on this and additional gels (Moller, unpublished). The picture presented here is from a single gel which originally contained also samples from 40 and 60 min treatments in each set. These were removed to make the final autoradiograph less cumbersome. In all cases these patterns were very similar to the 50 min samples in each set.


Pretreatment +30 minutes at 40.2°C
Pretreatment (34°C)
+0 hr
+1 hr
+3 hr
+7 hr
C 5' 15' 30' 50' 70' 80' 0 5' 15' 30' 50' 70' 80' 0 5' 15' 30' 50' 70' 80' 0 5' 15' 30' 50' 70' 80'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
83.4
76.6
75.5
74
72.7
72
70
67.7
59.9-51.8
47.2
46.3
45.3
43.9
42.4
36.7
27.5
26.8
22.4
21

just as remarkable that relatively little translation is evident in the 50, 70, and 80 min samples. If, for example, one compares the protein synthesis exhibited in tracks 4 and 5 with the corresponding samples in tracks 11 and 12 it would appear that the second and higher temperature shock either caused much more degradation of messenger RNA in the 50 min sample than in the 30 min sample or much more of the 50 min messenger was rendered inaccessible to translation. The same is true for the 70 and 80 min samples. Degradation of messenger seems unlikely from a consideration of the results shown in the 1 hr and 3 hr series. Here the 50-80 min samples (tracks 19-21 and 26-28) show a reappearance of strong synthesis of the major heat shock proteins. These results require either extensive new transcription in the 1 and 3 hr series or reactivation of message stored in a masked form during the pretreatment phase at 34°C.

Protection and RNA Synthesis

We presented evidence earlier (Mitchell and Lipps, 1978) that a high temperature (40.2°C) shuts down both translational and transcriptional activities. It was shown further that in this situation protein synthesis resumes before general RNA synthesis. These experiments were done on thoracic hypoderm tissue from 34 hr pupae and the results anticipated the suggestions made here regarding the protein synthesis patterns in Figure 4. That is, the initial resumption of protein synthesis after it has been turned off by a heat shock can be accounted for if the conditions favor storage of mRNA in a form that can be reactivated. Additional evidence that supports this conclusion is shown in Figure 5. Parts B, C, and D of Figure 5 are from autoradiographs (scanning electron microscope) of the tip of the left arm of chromosome 3 (as a representative region) as shown in part A. The preparations were made from a 10 min pulse labeling of late third instar salivary glands of

Figure 5. Transcriptional Activity on Salivary Gland Chromosomes

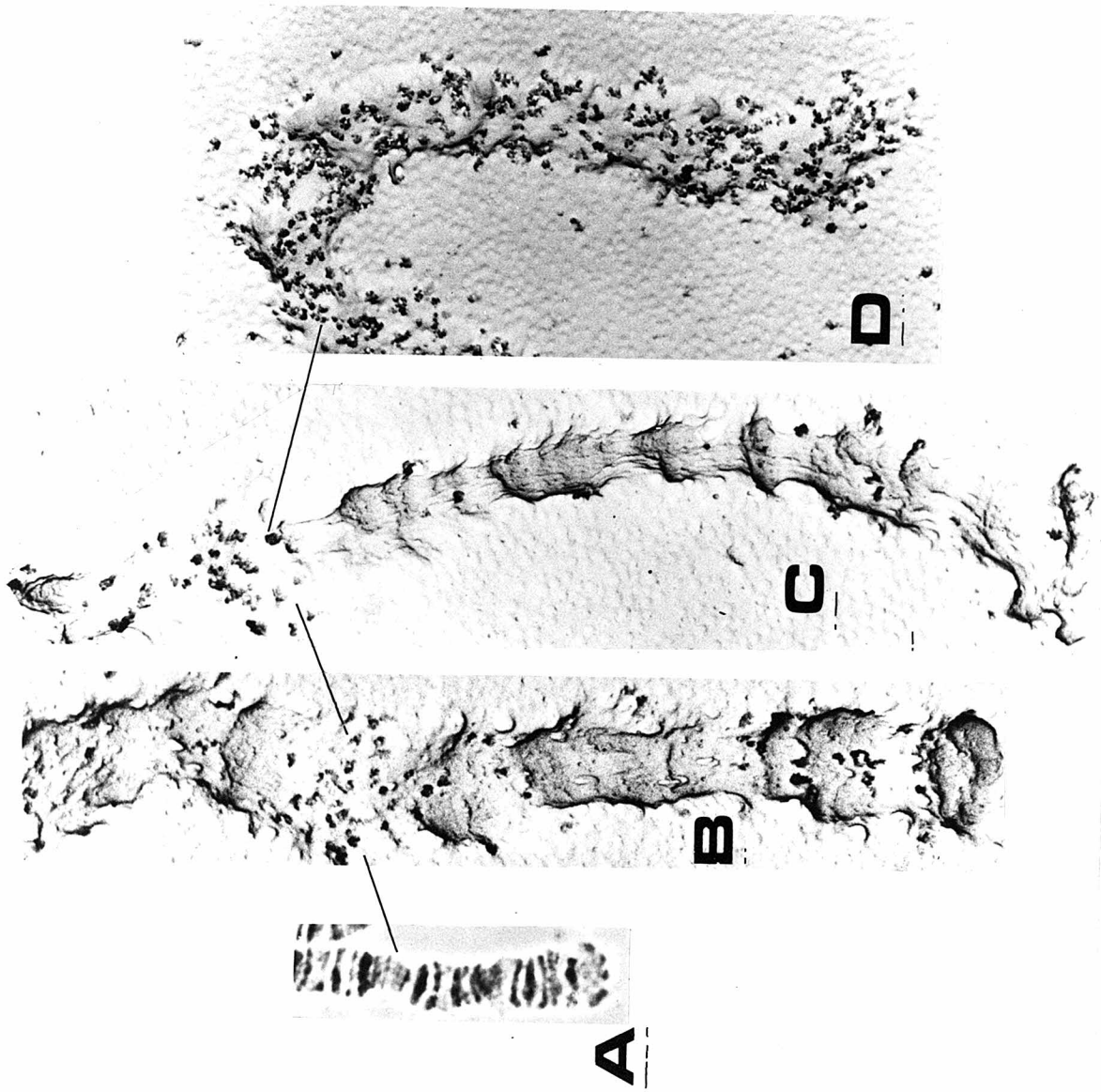
Part A shows a light microscope picture of the tip of a normal 3 L salivary gland chromosome while B, C, and D show autoradiographs of corresponding chromosome sections as revealed in the scanning electron microscope photographs. The connecting line shows the position of the heat shock puff designated 63BC.

B. An autoradiogram from a gland from a larva which received a 50 min pretreatment prior to a 30 min shock at 40.2°C.

C. An autoradiogram from a gland from an animal which received a 30 min heat shock at 40.2°C.

D. An autoradiogram from a gland from an animal which was kept at 25°C.

All labeling was done for 10 min at 25°C using ^{3}H-uridine and isolated salivary glands.


D
C
B
A

Drosophila melanogaster and the conditions used were like those described for the protein synthesis results shown in Figure 4. Specifically picture D at the right corresponds to the 25°C control in track 1 (Figure 4). This general distribution of silver grains is typical for a chromosome region where there are no prominent puffs (Mitchell et al., 1978). Picture C is from pulse labeled salivary glands from larvae given a heat shock of 40.2°C for 30 min (track 15, Figure 4). Picture B is from glands from animals given a 50 min treatment at 34°C followed by a 30 min shock at 40.2°C and then a 60 min incubation at 20°C prior to the pulse label (conditions for track 19, Figure 4).

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

Discussion

It appears now that the molecular basis of phenocopy formation, as a result of heat shock, may be understood most clearly as part of a general pattern of dynamic interactions which involve simultaneously transcription, translation, and translation products. Although speculative in some aspects the pattern shown in Figure 6 fits the existing information remarkably well and it is presented as a framework for discussion.

Protection for Survival

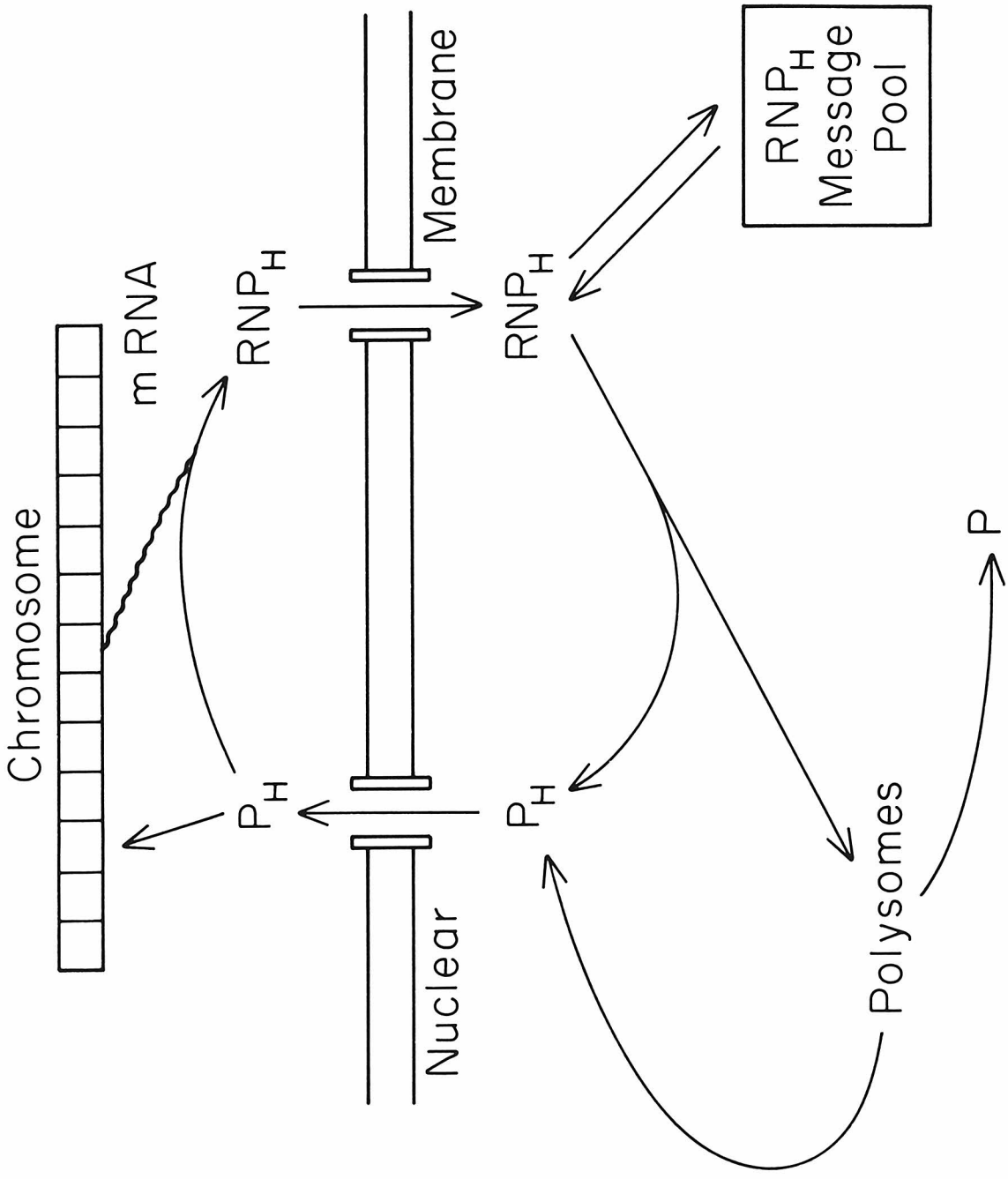
The protection phenomenon in which survival of heat shocked animals or cells can be enormously enhanced by a mild pretreatment at elevated temperature evidently depends on the simultaneous production of normal mRNAs and heat shock mRNA(s) plus heat shock protein(s) (Figures 1 and 4). A straightforward picture that describes the overall interactions is that shown in Figure 6. Beginning at the chromosome level we envisage that the preheat treatment induces production of heat shock messages in addition to the normal complement that is already in production. Then, all of these messages are exported as RNPs to the cytoplasm where they can go to polysomes for translation or enter into an inactive pool. Finally, as increased amounts of heat shock protein(s) is synthesized, transcription is more and more repressed and more and more messages are sequestered in the RNP pool. It is this gradual process which is essential in the pretreatment since a sudden shock at a high temperature represses all transcription quickly without allowing message accumulation. Furthermore, it is this accumulated and inactive message that provides the basis for survival after a drastic shock and it is reasonable to think that the message pool provides the memory for resumption of development in the pattern that existed when the heat shock was applied. These various presumptions all have a sound basis in the data presented earlier (Mitchell and Lipps, 1978) and in the information here in Figures 4 and 5. In addition we have shown in preliminary experiments that heat shock proteins appear in abundance in sucrose gradient fractions which contain polysomes and RNPs in preference to other proteins labeled at the same time. Further details will be presented elsewhere.

Specific Protection and Phenocopies

We presented evidence earlier (Mitchell and Lipps, 1978) implicating the failure

Figure 6. A Diagram to Illustrate a Dynamic Mechanism that may Explain the Pretreatment-Protection Phenomenon

P_H is used to indicate one or more heat shock proteins and RNP_H ribonucleoproteins in which heat shock proteins are involved. It is not intended that the heat shock proteins exclusively are involved in the transport cycle or in maintaining a message pool.


Chromosome
mRNA
RNP$_H$
Membrane
RNP$_H$
RNP$_H$ Message Pool
P$_H$
P$_H$
Nuclear
Polysomes
P

of transcriptional activity at a specific genetic locus as the primary step in the production of a specific phenocopy as the result of heat shock. The present information (Figures 2 and 3) gives strong support to this concept. Protection against production of the phenocopy of hook by a pretreatment occurs only during the sensitive period of development when the phenocopy is induced without the pretreatment. The protection then can be considered to be due to accumulation and storage of a specific message that is normally transcribed only during the sensitive period. Here too as with survival protection it is reasonable to assume that a drastic heat shock without a pretreatment simply shuts down all transcription too quickly to trap a significant quantity of specific message. We have considered previously that a phenocopy results when development proceeds before recovery of a given specific transcriptional activity. The results given here are in accord with this assertion but the existence of a message pool of variable size can complicate the picture.

General Considerations

The pattern of dynamic interactions which relate transcription, mRNA transport, and protein synthesis as depicted in Figure 6 is not greatly different from such relations implied by the work of a number of earlier investigations (Perry and Kelley, 1968; Spirin, 1969; Lukanidin et al., 1972; Blobel, 1973; and see also reviews by Perry et al., 1973, Lewin, 1974, and Spirin, 1978). It is also quite compatible with the more recent observations of Kelley and Schlesinger (1978) on the effects of heat shock on chick embryo fibroblasts and the findings of Jain and Sarkar (1979) on the ribonucleoprotein particles of chick embryo muscles.

Particularly pertinent in relation to our results is the earlier report of Schochetman and Perry (1972) in which an apparent direct relation was shown

between heat treatments and RNP accumulation in L cells. The evidence presented here supports both the existence of a cytoplasmic pool of RNPs and the postulate that such RNPs are of fundamental importance in the regulation of development. We have added the suggestion of a direct involvement of heat shock protein(s) in the system both in the nucleus (Mitchell and Lipps, 1975) and in the cytoplasm but this may be only part of the normal picture. It is of interest to note that special heat shock proteins are induced in mammalian and avian cells just as they are in Drosophila (Kelley and Schlesinger, 1978) but in these situations also it remains to be established just how the heat shock proteins function.

Our evidence (see Figure 4) suggests that RNPs as they are exported from the nucleus are different from RNPs accumulated in an inactive pool. Thus, as represented in Figure 6 emerging RNPs can go directly to polysomes or to an inactive pool. This concept allows for potential regulatory functions concerned with RNPs at the levels of insertion into and retrieval from a masked state. If indeed this is a standard practice in all cells then the unique phenomenon concerning masked message in oocytes (Crippa et al., 1967) is not the accumulation of message but the failure of protein synthesis.

Experimental Procedures

Fly Stocks

A single Oregon R wild stock was used for all the experiments described in this paper. This stock is a little more heat resistant than the Ore-R, Urbana S, and Canton-S cultures used previously. In this case 41.3°C for 40 min is about equivalent in survival to the 40.2°C for 40 min applied in earlier experiments (Mitchell and Lipps, 1978).

Details of culture and selection of animals in developmental synchrony were described earlier (Mitchell and Mitchell, 1964; Mitchell and Lipps, 1978).

Heat Treatments

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

Labeling

Labeling of salivary glands using three pairs each in 4 µl of ^{35}S-methionine (Sp activity 600) or ^{3}H-uridine (Sp activity 50), each containing 10 µCi, was carried out at 25°C in MOPS buffer at pH 7.0 (Tissieres et al., 1974).

Gel System

The SDS acrylamide gel used for these experiments was a 10 to 20% exponential gradient gel prepared as described by Van Blerkom and Manes (1974) and modified by Moller (1979).

Scanning Electron Microscope

Samples for observing bristle morphology were prepared as described by Mitchell and Lipps (1978) and those for observing chromosome autoradiographs were made as described earlier (Mitchell and Lipps, 1975; Mitchell et al., 1978).

Acknowledgments

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References

Blobel, G. (1973). Protein of MWT 78,000 bound to a polyadenylate region of eucaryotic messenger RNA. Proc. Nat. Acad. Sci. USA 70, 924-928.

Chomyn, A., Moller, G., and Mitchell, H. K. (1979). Patterns of protein synthesis following heat shock of pupae. Dev. Genet., in press.

Crippa, M., Davidson, E. H., and Mirsky, A. E. (1967). Persistence in early amphibian embryos of informational RNAs. Proc. Nat. Acad. Sci. USA 57, 885-892.

Jain, S. K., and Sarkar, S. (1979). Poly(riboadenylate)-containing messenger ribonucleoprotein particles of chick embryo muscles. Biochemistry 18, 745-753.

Kelley, P. M., and Schlesinger, M. J. (1978). The effects of amino acid analogues and heat shock on gene expression in chick embryo fibroblasts. Cell 15, 1277-1286.

Lewin, B. (1974). Protein associated with RNA. In Gene Expression, 2 (John Wiley and Sons, New York) pp. 250-256.

Lukanidin, E. M., Zalmanzon, E. S., Komaromi, L., Samarina, O. P., and Georgiev, G. P. (1972). Structure and function of informers. Nature New Biol. 238, 193-197.

Milkman, R. (1962). Temperature effects on day old Drosophila pupae. J. Gen. Physiol. 45, 777-799.

Milkman, R. (1966). Analyses of some temperature effects on Drosophila pupae. Biol. Bull. 131, 331-345.

Mitchell, H. K. (1966). Phenol oxidases and Drosophila development. J. Insect Physiol. 12, 755-765.

Mitchell, H. K., Chen, P. S., Lipps, L. S., and Moller, G. (1978). Separation of Drosophila RNAs on acrylamide gels in formamide. Insect Biochem. 8, 29-35.

Mitchell, H. K., and Lipps, L. S. (1975). Rapidly labeled proteins of the salivary gland chromosomes of Drosophila melanogaster. Biochem. Genet. 13, 585-602.

Mitchell, H. K., and Lipps, L. S. (1978). Heat shock and phenocopy induction in Drosophila. Cell 15, 907-918.

Mitchell, H. K., and Mitchell, A. (1964). Mass culture and age selection in Drosophila. Dros. Inf. Serv. 39, 135.

Moller, G. (1979). Ph.D. Thesis, California Institute of Technology, Pasadena, California.

Perry, R. P., Greenberg, J. R., Kelley, D. E., LaTorre, J., and Schochetman, G. (1973). Messenger RNA: its origin and fate in mammalian cells. In Gene Expression and Its Regulation, 1, Kenney et al., eds. (Plenum Publishing Co.) p. 149.

Perry, R. P., and Kelley, D. E. (1968). Messenger RNA-protein complexes and newly synthesized ribosomal subunits. J. Mol. Biol. 35, 37-60.

Petersen, N. S., Riggs, A. D., and Seecof, R. L. (1977). A method for establishing cell lines from Drosophila melanogaster. In Vitro 13, 36-40.

Schochetman, G., and Perry, R. P. (1972). Characterization of the mRNA released from L cell polyribosomes as the result of temperature shock. J. Mol. Biol. 63, 577-590.

Spirin, A. S. (1969). Informosomes. Eur. J. Biochem. 10, 20-35.

Spirin, A. S. (1978). Review on informosomes. Nucleic Acids Res. and Mol. Biol. 21, 1-38.

Tissières, A., Mitchell, H. K., and Tracy, U. M. (1974). Puffs and protein synthesis in Drosophila. J. Mol. Biol. 84, 389-398.

Van Blerkom, J., and Manes, C. (1974). Development of preimplantation rabbit embryos in vivo and in vitro. 2. Comparison of qualitative aspects of protein synthesis. Dev. Biol. 40, 40-51.