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Heat shock of *Drosophila melanogaster* induces the synthesis of new messenger RNAs and proteins

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The heat shock proteins, labelled *in vivo* with [^{35}S]methionine, were separated by sodium dodecylsulphate–polyacrylamide gel electrophoresis and fingerprinted after tryptic digestion. Eight distinct heat shock polypeptides were characterized in this way.

Heat shock messenger RNAs were isolated and partially purified. Assayed *in vitro* for protein synthesis, they were found to code for heat shock polypeptides.

Some parameters of the kinetics of *in vivo* synthesis of the heat shock proteins are presented.

Introduction

When *Drosophila melanogaster* normally raised at 25 °C is exposed to a temperature of 37 °C, it appears that a series of specific genes are activated, while most of the other genes, active at 25 °C, are repressed. After this heat shock, 9–10 new puffs are seen on the giant chromosomes of the salivary glands (Ritossa 1962; Ashburner 1970) accompanied by RNA synthesis at the sites of these puffs (Tissières, Mitchell & Tracy 1974). Moreover most of the other puffs, active at 25 °C before the heat shock, rapidly regress at the higher temperature (Ashburner 1970). At the same time, the rapid synthesis of a small number of new proteins is observed, while the rate of synthesis of most cellular proteins, actively made before the heat shock, is strongly reduced (Tissières *et al.* 1974). New species of RNA are synthesized at the higher temperature and hybridize *in situ* to the heat shock puffs (McKenzie, Henikoff & Meselson 1975; Spradling, Penman & Pardue 1975). In the work described here, the new gene products synthesized after the heat shock were further characterized. The heat shock proteins, analysed by tryptic fingerprint, were found to consist of eight distinct polypeptides. Heat shock specific messenger RNAs were isolated, and partly purified. Added to an *in vitro* system for protein synthesis, they were shown to be translated into the heat shock induced polypeptides.

Materials and methods

Tissue culture cells and flies

The *Drosophila melanogaster* tissue culture cell line KC 161 was received from Dr Echalier. It is a subline of the KC line previously established by Echalier & Ohanessian (1969). These cells were adapted to grow in suspension in D22 medium (Echalier & Ohanessian 1970) containing 2% foetal calf serum (serum screened for viruses and mycoplasma, Gibco). The cells, grown in spinner flasks at 25 °C, had a generation time of about 24 h and were maintained at

concentrations between 2×10^6 and 8×10^6 cells/ml. Wild-type *Drosophila melanogaster* (Kolmar) was raised at 25 °C.

[^{35}S]*methionine labelling*

Tissue culture cells, washed and concentrated tenfold in D22 medium without methionine and serum, were labelled for 1 h with 40 μCi/ml [^{35}S]methionine (New England Nuclear, 300–500 Ci/mmol) at 37 °C (heat shock) or 25 °C (control). Salivary glands were labelled essentially as described by Tissières *et al.* (1974) in modified Grace's (1962) medium (1 part of 10 % ethanol added to 5 parts of medium without methionine). Proteins were extracted by boiling for 3 min in electrophoresis sample buffer.

Sodium dodecylsulphate–polyacrylamide gel electrophoresis

Proteins were separated by discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS) using the system of Laemmli (1970) in vertical slab gels (Studier 1972) ($0.16 \times 15 \times 15$ cm) and detected by autoradiography or fluorography of the dry gel according to Laskey & Mills (1975). Apparent molecular masses were determined in SDS gels of the following concentrations: 15, 12.5, 10 and 7.5 % acrylamide and 0.4, 0.33, 0.27 and 0.2 % *bis*-acrylamide respectively.

Tryptic fingerprint analysis

Tryptic peptides prepared as described by Allet, Katagiri & Gesteland (1973) were separated by two dimensional chromatography (first dimension: pyridine–isoamylalcohol–water (7:7:6) (Adams & Capecchi 1966); second dimension: butanol–pyridine–acetic acid–water (5:4:1:4)). Radioactive spots were detected by autoradiography.

RNA preparation and purification

In order to isolate heat shock mRNA, exponentially growing cells were collected by centrifugation and gently resuspended in complete medium prewarmed at 37 °C at a concentration of about 10^8 cells/ml. The cells were kept in a water bath at 37 °C under gentle agitation for 80 min. To label the RNA, [5,6-^{3}H]uridine (New England Nuclear, more than 30 Ci/mmol) was added to a concentration of 30 μCi/ml to the cells 15 min after the temperature shift. After 80 min at 37 °C, the cells were quickly chilled to 0 °C by rapid stirring of the flask in an ice–methanol cooling mixture. The heat shocked cells were then collected by centrifugation at 2 °C. The control cells, kept at 25 °C, were collected at room temperature, and the cell pellets were resuspended and washed twice in ice-cold isotonic phosphate saline buffer.

The cells were lysed by the procedure of McKenzie *et al.* (1975) in 4 volumes of lysis buffer, composed of 0.05 M Tris–HCl, pH 7.4, 0.25 M KCl, 0.05 M $MgCl_2$, 25 mg/ml of heparin and 0.2 % Triton X-100 (Sigma). After centrifugation for 10 min at 16000 *g*, 3–4 ml fractions of the supernatant were layered on 34 ml linear sucrose gradients (0.5–1.5 M sucrose in 0.05 M Tris–HCl, pH 7.4, 0.25 M KCl and 0.025 M $MgCl_2$ and centrifuged in a Beckman SW27 rotor for 105 min at 27000 rev/min and 2 °C. The gradients were collected from the bottom of the tube in 1.5 ml fractions. Aliquots were removed to measure the absorbance at 260 nm and the trichloroacetic acid (TCA) precipitable radioactivity. The polyribosomes and ribosomes were quantitatively precipitated by addition of one volume of ethanol at −20 °C. After 2 h at −20 °C, the precipitate was collected by centrifugation at 20000 *g* for 30 min at −10 °C. The RNA was then prepared by phenol extraction according to Aviv & Leder (1972). After

two ethanol precipitations, the RNA was fractionated on oligo(dT_{12-18}) cellulose into poly(A)$^+$ and poly(A)$^-$ RNA according to Spradling *et al.* (1975). The RNA was heated for 3 min at 65 °C and centrifuged for 5 min at 10000 *g* before loading on the column. Flow-through RNA (poly(A)$^-$) and low-salt-eluted RNA (poly(A)$^+$) were precipitated with 3 volumes of ethanol in the presence of 0.2 M NaCl, left overnight at −20 °C, dissolved in 0.01 M Tris, pH 7.4, and 0.1 % SDS and stored at −70 °C.

RNA was analysed by centrifugation in low-salt sucrose gradients after heat denaturation according to Haines, Carey & Palmiter (1974), using either SW40 or SW56 rotors in a Beckman L65B ultracentrifuge.

Electrophoresis of RNA in polyacrylamide gels was performed according to Mirault & Scherrer (1971) in 14 cm gels in quartz tubes 6 mm in diameter, at room temperature and without deoxycholate. The gels, of uniform polyacrylamide concentration, containing 10 % glycerol, were electrophoresed at room temperature, frozen and cut into 1.5 mm slices. The RNA was eluted from the gel by shaking each slice in 0.5 ml 0.01 M triethanolamine–HCl, pH 7.4, and 0.1 % SDS at 4 °C for 48 h. After removal of the supernatant the elution step was repeated once. Aliquots were removed to measure the TCA-precipitable radioactivity. The yield of elution was consistantly high (80–90 %) with slight variations depending on the size of the RNA and the gel concentration. Poly(A)-containing RNA was purified and recovered from oligo(dT) cellulose. The RNA was essentially quantitatively recovered as poly(A)$^+$ RNA. This indicates that little if any RNA degradation had occurred during elution from the gel.

All buffers used for the preparation of RNA were autoclaved after addition of 0.01 % diethylpyrocarbonate.

In vitro *translation*

To remove SDS from mRNA, the samples were precipitated with 3 volumes of ethanol. The RNA pellets were dissolved in 0.2 M sodium acetate, pH 5, and reprecipitated with ethanol. The final pellets were rinsed twice with 75 % ethanol, dried in vacuum and the RNA was dissolved in sterile double-distilled water and stored at −70 °C.

The cell-free translation system was the messenger dependent rabbit reticulocyte lysate (m.d.l.) described by Pelham & Jackson (1976). The reaction mixture consisted of 20 µl complete m.d.l., containing 5 µCi [^{35}S]methionine at 300–500 Ci/mmol, 1 or at most 2 µl of RNA, or water in the case of the blank, were added. Incubation was carried out at 30 °C for 20–60 min. The rate of incorporation of [^{35}S]methionine was found to be linear for at least 20 min. The decrease which followed depended on the RNA preparation used. Translations *in vitro* were carried out with limiting RNA concentrations.

For electrophoretic analysis, 100 µl of electrophoresis sample buffer was added to each reaction mixture. After 3 min in a boiling water bath, 5–10 µl of the total sample were loaded for the electrophoresis on slab gels as described above.

Results

(*a*) *Heat shock proteins*

Cells in culture or salivary glands were pulse labelled with [^{35}S]methionine, both at 25 °C and after a heat shock at 37 °C, and figure 1 shows the pattern obtained when the proteins, separated by electrophoresis in SDS-polyacrylamide gels, are detected by autoradiography.

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New protein bands appear after heat shock while the synthesis of most of the proteins synthesized at 25 °C is strongly reduced, as was observed earlier (Tissières *et al.* 1974; McKenzie *et al.* 1975; Lewis, Helmsing & Ashburner 1975). The overall rate of incorporation of [^{35}S]methionine is roughly the same at the two temperatures. The effect obtained with tissue culture cells is qualitatively similar to that seen with salivary glands. However, in the glands, the 84000 molecular mass band is labelled less than the 70000 band, than is the case in the tissue culture cells. In the latter, the small molecular mass proteins (22000–27000) are more labelled after 2 h at 37 °C (see figure 1) than earlier during the heat shock.

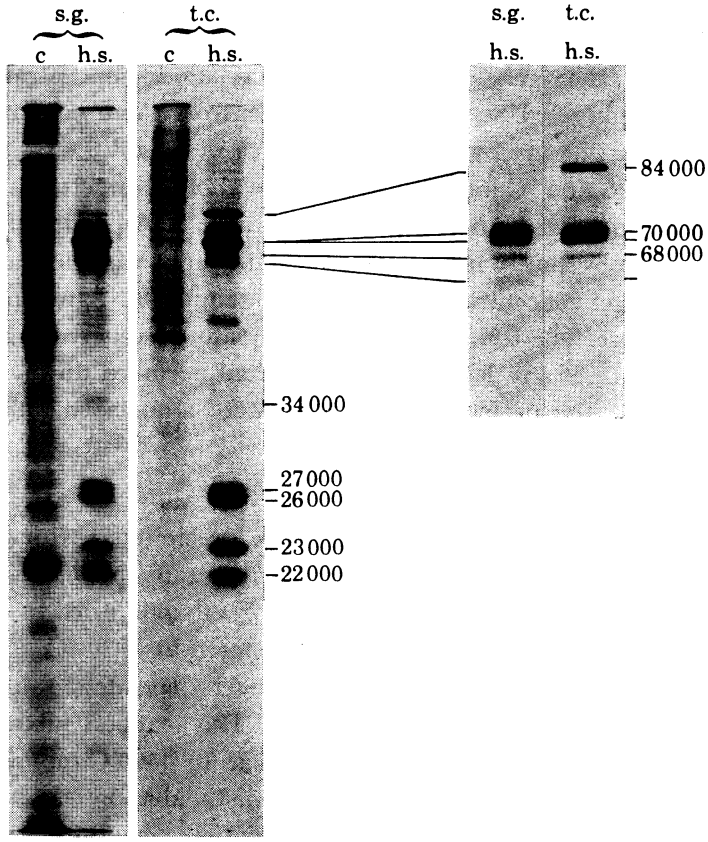

FIGURE 1. The effect of heat shock on the gel electrophoresis autoradiograph pattern of [^{35}S]methionine labelled proteins from tissue culture cells and salivary glands. Tissue culture cells (t.c.) were labelled for 1 h at 37 °C following a heat shock (h.s.) of 2 h at the same temperature. Control (c) cells were labelled in a parallel incubation at 25 °C. Salivary glands (s.g.) were labelled after heat shock as previously described (Tissières *et al.* 1974). Control (c) glands were labelled in a parallel incubation at 25 °C. The proteins were separated by SDS–polyacrylamide gel electrophoresis and detected by autoradiography of the dried gels. The concentrations of the gels and the conditions of electrophoresis were: at the left, 12.5% acrylamide, 0.33% *bis*-acrylamide, and 50 V for 17 h; at the right, 15.0% acrylamide, 0.09% *bis*-acrylamide, and 130 V for 17 h. The apparent molecular masses were determined as indicated in Materials and Methods.

The apparent molecular masses of the heat shock polypeptides were determined from their migration in SDS–polyacrylamide gels of different concentrations, calibrated with standards of known molecular mass (see Methods) and the results are given in figure 1. Several heat shock proteins migrated somewhat anomalously, which could be due to chemical modifications. Thus the molecular masses should be considered as nominal values.

Analysis of tryptic digests of heat shock proteins

How many distinct heat shock polypeptides are there? To answer this question, labelled protein bands from heat shocked salivary glands or cells in culture were eluted from the gels, oxidized with performic acid and digested with trypsin. After separation by two dimensional chromatrography, the methionine labelled peptides were detected by autoradiography. The 84000, 70000, 68000, 34000, 27000, 26000, 23000 and 22000 molecular mass bands all gave clearly different fingerprints indicating that each of these bands represents a distinct polypeptide. Typical results for the proteins of molecular masses 84000, 70000, 23000 and 22000 daltons are shown in figure 2.

In addition to the 68000 and 70000 molecular mass polypeptides, other bands can be resolved in the 65000–70000 range (figure 1). The corresponding fingerprints were found to be similar to those of the 70000 species, suggesting that these bands share closely related primary structures.

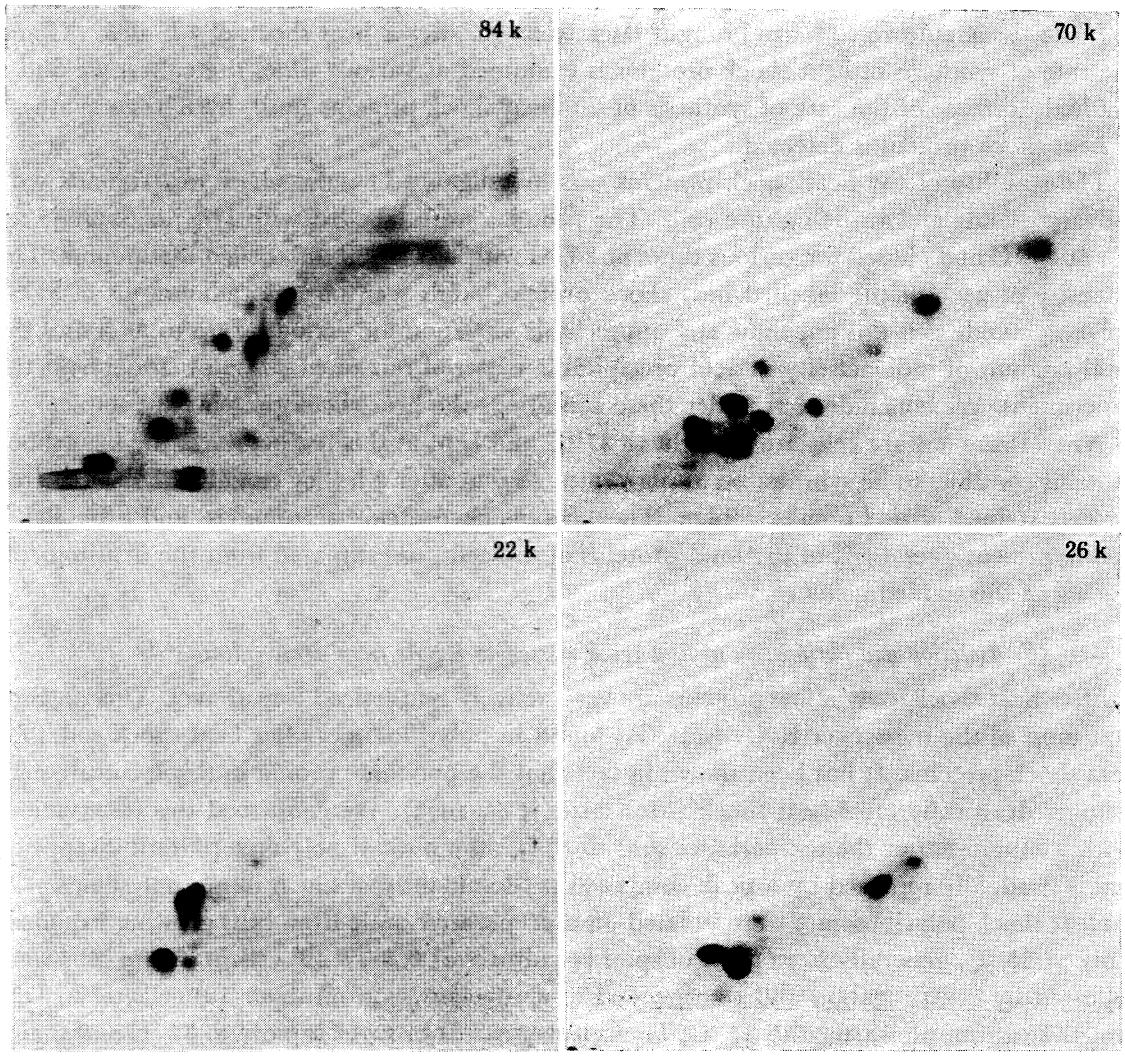

FIGURE 2. Tryptic fingerprints of the 84000, 70000, 26000 and 22000 molecular mass heat shock polypeptides from tissue culture cells labelled *in vivo* with [^{35}S]methionine.

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The proteins from salivary glands and tissue culture cells gave identical fingerprints for each of the eight heat shock proteins.

Kinetics of synthesis of heat shock proteins

A pulse labelling of at least 10 min with [^{35}S]methionine is necessary to detect the appearance of the heat shock proteins with either salivary glands or tissue culture cells. Within this time, synthesis of all heat shock proteins is initiated, so that no definite sequence in their appearance can be observed, in contrast to the observation of Lewis *et al.* (1975) with *Drosophila hydei*. After about 60 min of heat shock with salivary glands, the intensities of the heat shock protein bands on the autoradiographs of the gels following pulse labelling indicate that the proteins are synthesized at maximum rate. The rate of synthesis declines thereafter to about 50 % after 3 h at 37 °C. With the cells in culture, the maximum rate of synthesis of the heat shock proteins is reached in 90–120 min. The subsequent decline in the rate of synthesis of the heat shock proteins is much less pronounced than with the salivary glands. This rate was found to reach about 50 % of its initial value after 8 h at 37 °C.

If the tissue culture cells are brought back to 25 °C after a heat shock of 1 h at 37 °C and the rate of synthesis of heat shock proteins is examined at various times thereafter, we find a gradual decrease of the rate of synthesis of the heat shock proteins until, by 8 h, no synthesis of heat shock protein is detected.

The stability of the heat shock proteins was investigated in pulse–chase experiments with salivary glands and tissue culture cells. The proteins were labelled with [^{35}S]methionine for 1 h at 37 °C and chased for various times at 25 °C with 200 μg/ml unlabelled methionine. The intensity of each of the labelled heat shock protein bands seen on autoradiographs of SDS-polyacrylamide gels did not show any appreciable variation for periods of up to 20 h and the total amount of radioactivity in acid precipitable material remained constant throughout the experiment. We conclude that under these conditions the heat shock proteins are stable.

When tissue culture cells are brought to 37 °C and kept at this temperature, the heat shock proteins continue to be synthesized at high rates, so that after 2 h they can already be detected on gels stained with Coomassie blue. The heat shock proteins accumulate and after 6–8 h they represent over 10 % of the total proteins of the cells, as estimated from the densitometer tracing of the stained gels.

(*b*) *Isolation and purification of heat shock messenger RNAs from tissue culture cells*

After heat shock, only a few proteins are very actively synthesized (see above). This suggests that most of the messenger RNA (mRNA) found in polyribosomes after heat shock codes for heat shock proteins. It has been shown in fact that the polysome profile in tissue culture cells changes drastically after heat shock (McKenzie *et al.* 1975). We confirmed this observation. Within minutes after the temperature shift to 37 °C, the normal polysome pattern disappears and is gradually replaced by a peak composed predominantly of much larger polysomes.

Heat shock polyribosomes were isolated on a preparative scale from cells growing exponentially at 25 °C, generally from 2–4 l of spinner cultures at 0.5–1 × 10^7 cells/ml. The cells were collected by centrifugation and resuspended to a concentration of about 10^8 cells/ml in D22 growth medium prewarmed to 37 °C. The cells were thus brought rapidly to 37 °C and maintained at that temperature for 80 min after which they were rapidly chilled on ice. Some experiments were done at 36 °C and gave similar results.

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For the large scale purification of heat shock mRNA, several successive but qualitatively equivalent preparations of polyribosomes were pooled. One of these preparations was labelled with [^{3}H]uridine for 60 min, starting 15 min after the temperature shift. Polyribosomes from control cells kept at 25 °C were prepared similarly in parallel experiments.

After the lysis of the cells (see Methods) the postmitochondrial supernatant was centrifuged in sucrose gradients in an SW27 rotor in a Beckman centrifuge. The sedimentation pattern obtained in a typical experiment is shown in figure 3. The gradients were divided into five

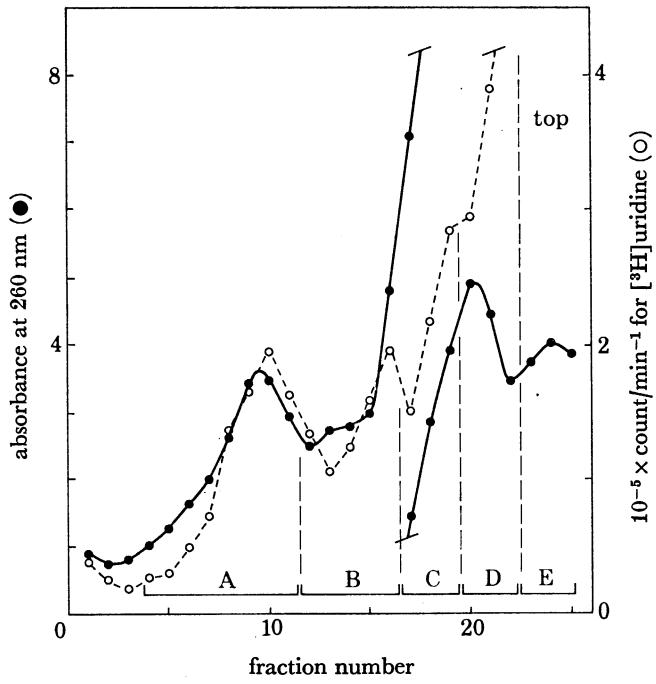

FIGURE 3. Preparative isolation of polyribosomes by sucrose gradient sedimentation. The cells were labelled at 37 °C from 15 to 75 min after the temperature shift, lysed and the postmitochondrial supernatant centrifuged in sucrose gradients in an SW27 rotor in a Beckman centrifuge at 27000 rev/min for 105 min at 2 °C. The gradient was collected from the bottom into 25 fractions of 1.5 ml each. Gradient fractions were pooled as shown in the figure. The scale for the absorbance is decreased fivefold from fractions 17–25.

fractions as follows: A, large polyribosomes; B, small polyribosomes; C, first ribosomal fraction; D, second ribosomal fraction, and E, top of the gradient. Fraction E mostly contains soluble proteins and labelled 4–5 *S* RNA which was not further characterized. RNA from each pool was prepared by phenol extraction (see Methods) and fractionated by chromatography on oligo(dT)-cellulose into poly(A)$^-$ and poly(A)$^+$ RNA. The poly(A)$^+$ RNA of the fractions A, B, C and D (see figure 3) contained respectively 52, 43, 14 and 5 % of the total label in each pool. These same fractions also contained respectively 3.9, 4.8, 2.2 and 2.2 % of the total absorbance at 260 mm in each fraction. The poly(A)$^+$ RNA label of the polysomal fractions A and B amounted to 88 % of the total poly(A)$^+$ label found in the gradient.

Almost all the label in poly(A)$^+$ RNA from the polyribosomes is displaced to slow sedimenting mRNPs by EDTA and has therefore the expected characteristics of mRNA (data not shown). Major contamination by nuclear RNA is therefore excluded. The poly(A)$^+$ RNA fractions are still contaminated with variable amounts of unlabelled ribosomal RNA, usually 1–2 % of total ribosomal RNA, which accounts for an appreciable fraction of the absorbance at

260 nm. Surprisingly, rechromatography of the poly(A)+ RNA fraction on oligo(dT)-cellulose did not significantly improve the purification. The poly(A)+ RNA fractions and the corresponding poly(A)− fractions were analysed by sedimentation on low salt SDS–sucrose gradients after heat denaturation according to Haines *et al.* (1974). Figure 4 shows the radioactive poly(A)+ RNA profile from large (*a*) and small (*b*) heat shock polyribosomes, compared with poly(A)+ RNA from equivalent polyribosome fractions derived from cells kept at 25 °C (*c*, *d*).

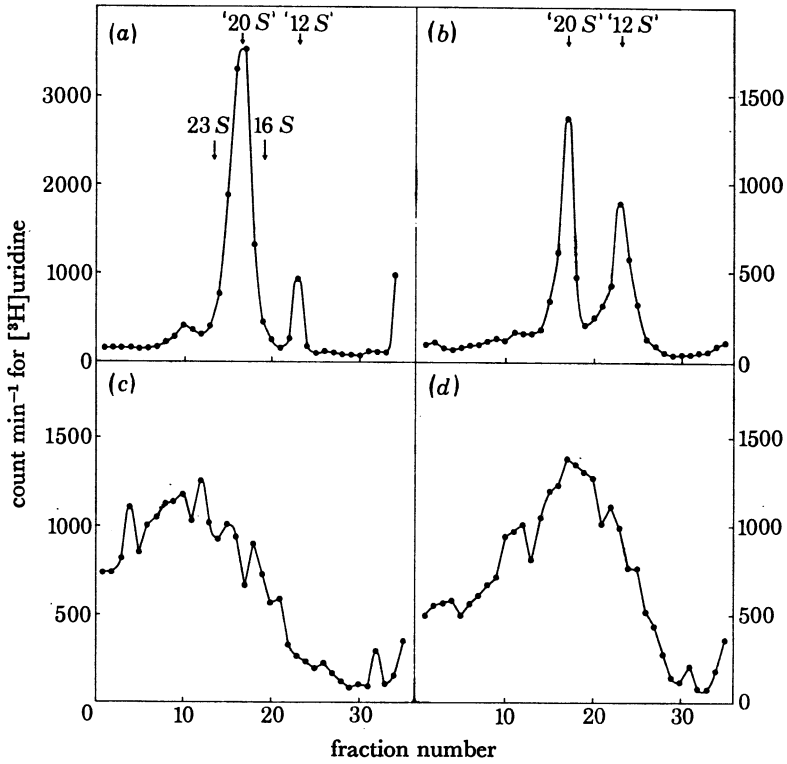

FIGURE 4. Sucrose gradient sedimentation of heat shock and control poly(A)+ RNA from large and small polyribosomes. RNA prepared from polyribosomes fractions A and B as shown in figure 3, from heat shocked and control cells maintained at 25 °C, was analysed by sedimentation in linear 5–20 % sucrose gradients according to Haines *et al.* (1974). Centrifugation was carried out for 150 min in a SW56 rotor at 56000 rev/min and 20 °C. (*a*) RNA from large heat shock polyribosomes (fraction A from figure 3); (*b*) RNA from small heat shock polyribosomes (fraction B from figure 3); (*c*) RNA from large *control* polyribosomes (corresponding to fraction A from figure 3); (*d*) RNA from small control polyribosomes (corresponding to fraction B from figure 3).

The labelled poly(A)+ RNA from large heat shock polyribosomes sediments predominantly at about 20*S*, while that of small heat shock polyribosomes sediments around 20 and 12*S*. These latter RNA classes are referred to as 20*S* RNA and 12*S* RNA respectively. In contrast, the poly(A)+ RNA from cells kept at 25 °C, which we shall call control RNA, sediments as expected quite heterogeneously throughout the gradient (figure 4*c*, *d*). Very little, if any, of this labelled control RNA is detected in labelled polysomal heat shock RNA (figure 4*a*, *b*).

The small amounts of labelled poly(A)+ RNA found in the ribosomal fractions (figure 3, fractions C and D) consist essentially of 20 and 12*S* RNA as in the pattern seen in figure 4*b*. The poly(A)− RNA from fractions A–D (figure 3) contains mostly unlabelled ribosomal RNA and labelled RNA sedimenting heterogeneously throughout the gradient (data not shown). The poly(A)− RNA fractions were not analysed further.

After sucrose gradient centrifugation, the poly(A)+ RNA was further fractionated by polyacrylamide gel electrophoresis under non-denaturing conditions (Mirault & Scherrer 1971). The gels were scanned at 260 nm, frozen and sliced, and the RNA eluted in low-salt SDS buffer (see Methods). The yield of elution varied from 80–90 % depending on the size of the RNA. Figure 5 shows the electrophoretic fractionation of the poly(A)+ 20*S* RNA isolated on sucrose gradients from large and small heat shock polyribosomes respectively. Most of the RNA (figure 5*a*) is found in one sharp peak, fraction II. This fraction migrates homogeneously in gels of different acrylamide concentrations from 2.5 to 3.5 %. Several small peaks are seen on either side. We know from other electrophoretic analyses, in which the RNA did not migrate so far in the gel, that 20*S* RNA does not contain any labelled RNA moving faster than that visible in figure 5. However, we observed that contaminating non-labelled ribosomal RNA has run off these gels. Note that 20*S* heat shock RNA from small polyribosomes is relatively enriched for the minor RNA species, fractions I and III, as seen in figure 5.

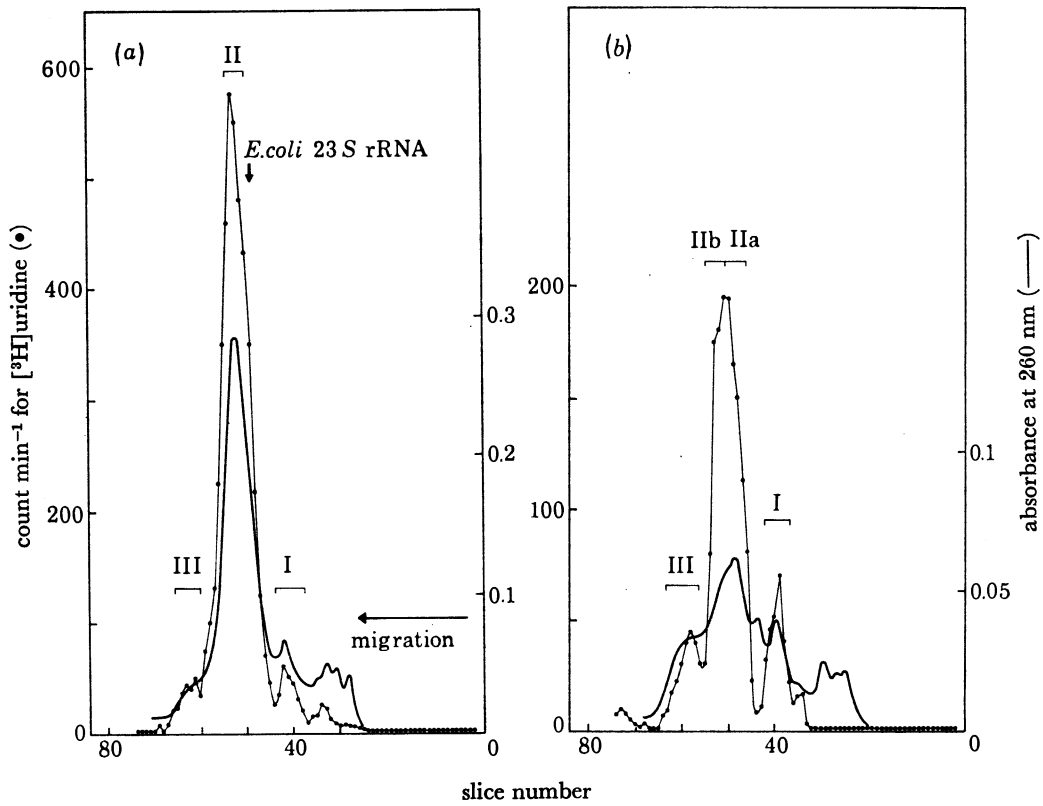

FIGURE 5. Electrophoretic pattern of 20*S* heat shock poly(A)+ RNA isolated on sucrose gradients as in figure 4 from the two polyribosome fractions A and B of figure 3. Electrophoresis was run for 19 h at 10 V/cm at room temperature with 3 % acrylamide. (*a*) 20*S* RNA from large heat shock polyribosomes; (*b*) 20*S* RNA from small heat shock polyribosomes. The TCA precipitable radioactivity given were from $\frac{1}{30}$ of the eluted RNA. *E. coli* 23*S* ribosomal RNA was run in a parallel gel as a reference marker.

Polyacrylamide gels in aqueous buffers do not allow accurate estimations of molecular weights. Under totally denaturing conditions in 98 % formamide gels run at 45 °C according to Spohr, Mirault, Imaizumi & Scherrer (1975), a large proportion of the 20*S* RNA still migrates between HeLa 18*S* RNA and *E. coli* 23*S* rRNA. Therefore the main component of

the 20*S* heat shock RNA (fraction II in figure 5) can be estimated to have a molecular mass of about 0.9×10^6.

The RNA eluted from the gels was pooled as shown in figure 5*a* and *b* in fractions I, II and III and repurified by chromatography through oligo(dT)-cellulose. More than 95% of the label still bound to the columns. In the case of the 20*S* main RNA component the final recovery was 0.24 A_{260} units, from a starting material of about 50 A_{260} units of large heat shock polyribosomes.

The total poly(A)$^+$ RNA from the small heat shock polyribosomes was also analysed by electrophoresis in non-denaturing gels. The pattern seen in figure 6 shows two labelled RNA fractions corresponding to the 20 and 12*S* peaks of the sucrose gradients from figure 4*b*. The 12*S* RNA migrates as a broad heterogeneous peak suggesting that there are several RNA species, poorly resolved. Nevertheless, 12*S* RNA fractions were pooled from the gel as shown in figure 6 in order to be assayed for messenger activity (see following section).

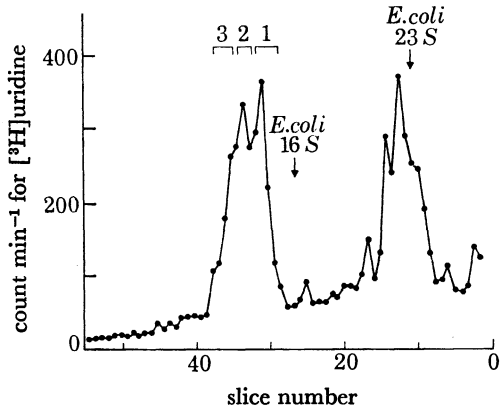

FIGURE 6. Electrophoretic pattern of poly(A)$^+$ RNA, from small heat shock polyribosomes as seen in figure 3, fraction B. Electrophoresis was run for 15 h at 10 V/cm at room temperature with 3.5% acrylamide. The TCA precipitable radioactivity ([^{3}H]uridine) was estimated on $\frac{1}{30}$ of each eluted RNA sample. *E. coli* 16 and 23*S* ribosomal RNAs were run in parallel gels as reference markers.

Recent experiments demonstrate that treatment of 12*S* heat shock RNA with oligo(dT) and RNase H, a procedure which removed the poly(A) tails (Vournakis, Efstratiadis & Kafatos 1975) and resolves the 12*S* RNA into three distinct labelled RNA bands after gel electrophoresis in urea. Work is in progress to purify these RNAs further by these means.

(*c*) In vitro *translation of heat shock messenger RNAs*

Pelham & Jackson (1976) have recently described a very efficient and sensitive messenger RNA dependent translation system from reticulocyte lysate. We used this system to assay RNA fractions for their messenger activity.

The poly(A)$^+$ RNA of the heat shock fractions A–D described in figure 3 were assayed for protein synthesis. The products of the reaction labelled with [^{35}S]methionine, were analysed by electrophoresis in SDS–polyacrylamide gels and the results are shown in figure 7. Messenger RNA from large heat shock polyribosomes directs predominantly the synthesis of a polypeptide of molecular mass 70000. Messenger RNA from small heat shock polyribosomes directs mainly the synthesis of polypeptides of approximate molecular masses 22000, 23000,

26000 and 27000, as well as 70000 and 84000. Thus, the main polypeptides made *in vitro* migrate in the gels at the position of the heat shock polypeptides made *in vivo*. Note, however, that other minor products are synthesized and that paradoxically more 84000 molecular mass protein is apparently synthesized by mRNA from small polysomes than by preparations from large polysomes.

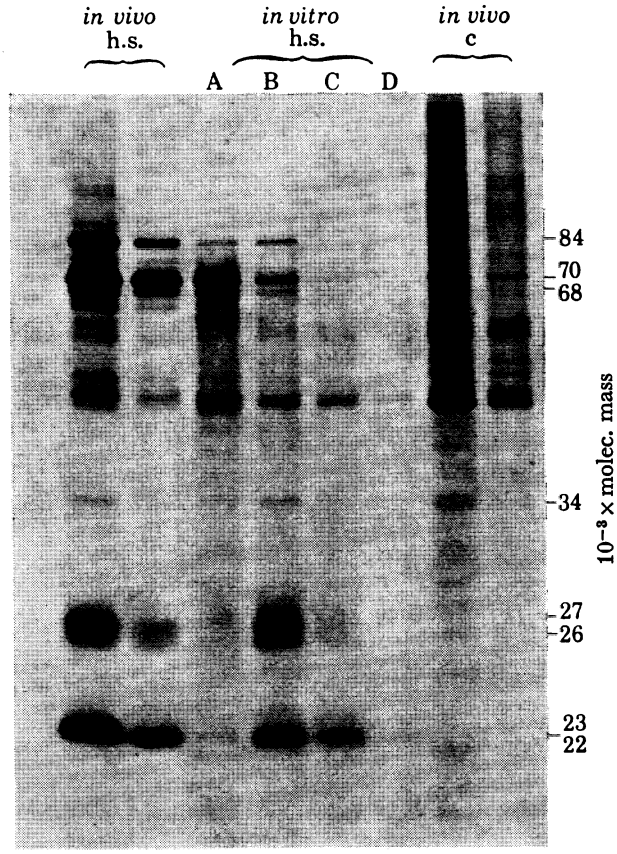

FIGURE 7. Translation of poly(A)$^+$ messenger RNA from polysomal fractions (see figure 3). Fluorogram of [^{35}S]methionine labelled polypeptides synthesized *in vitro* and *in vivo*, separated by electrophoresis in SDS–polyacrylamide gels. *In vivo* labelled heat shock polypeptides are seen in the second and third tracks at two different concentrations (ratio 5:2). *In vivo* labelled control polypeptides (at 25 °C) are seen in the eighth and ninth tracks at two different concentrations (ratio 5:2). Polypeptides synthesized *in vitro* by poly(A)$^+$ heat shock mRNA from fractions A, B, C and D (figure 3) are seen in the centre of the figure. Translation reactions were run for 20 min at 30 °C with 0.57, 0.7, 0.71 and 1.1 μg of RNA corresponding respectively to 0.25% of each fraction A, B, C and D. The translation product labelled *in vitro* in the absence of RNA (blank) is seen at the left in the figure. The reaction mixtures (see Methods) were diluted with electrophoresis sample buffer to 120 μl. Analysis by electrophoresis of 3 μl of each sample was carried out in a 15% polyacrylamide gel with 0.2% *bis*-acrylamide (see Methods). Fluorographic exposure was for 3 days at −70 °C.

Polysomal mRNA from control cells kept at 25 °C directs the synthesis of a large number of polypeptides, most of which migrate in the 25000–60000 molecular mass range. We do not observe clear bands at molecular masses 70000, 27000, 26000, 23000 and 22000, the positions of heat shock proteins, but a low level of synthesis of these polypeptides cannot be excluded. This suggests that mRNAs for heat shock polypeptides are either absent, or present in small amounts, in cells grown at 25 °C. These *in vitro* translation reactions were carried out for short times and at limiting mRNA concentrations.

The polysomal heat shock mRNA was further purified by centrifugation in sucrose gradients (see preceding section); fractions therefrom were translated *in vitro* and the results are shown in figure 8. The 20*S* heat shock mRNA (figure 4*a*) directs the synthesis of at least three polypeptides which migrate with molecular masses of 84000, 70000 and 68000, at the positions of the heat shock polypeptide synthesized *in vivo*. Small amounts of lower molecular mass bands are also seen. The 12*S* heat shock mRNA (figure 4*b*) directs the synthesis of at least three polypeptides which migrate with molecular masses of 26000–27000, 23000 and 22000 at the position of the heat shock polypeptides labelled *in vivo*. The 26000 and 27000 molecular

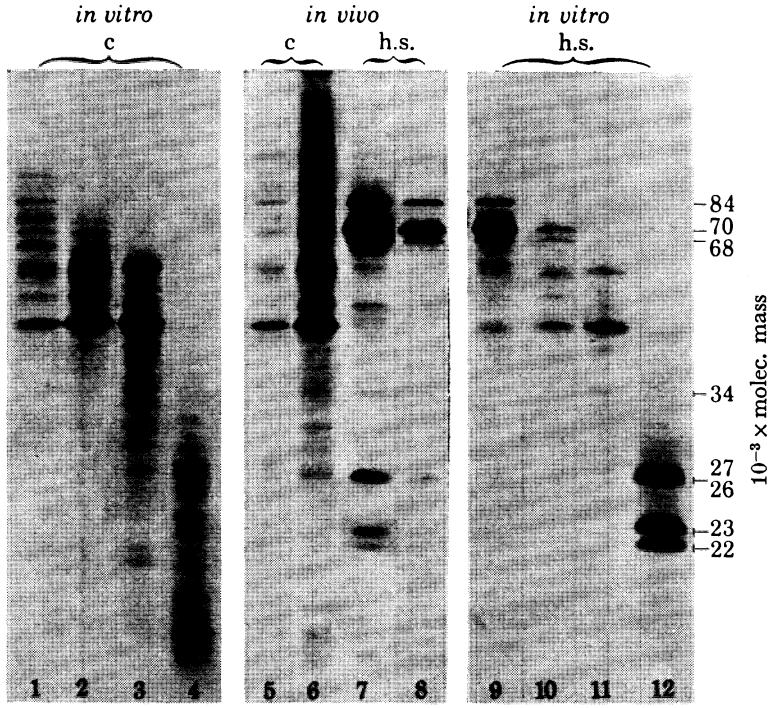

FIGURE 8. Fluorogram of [^{35}S]methionine labelled polypeptides synthesized *in vitro* by poly(A)$^+$ mRNA fractions from sucrose gradients (as in figure 4). In the centre panel, the polypeptides labelled for 1 h *in vivo* in control cells, at 25 °C (tracks 5 and 6) or in cells heat shocked for 1 h at 37 °C (tracks 7 and 8) are seen at two different exposures (5 h and 19 h). Polypeptides synthesized *in vitro* by 20, 18, 15 and 12*S* poly(A)$^+$ mRNA fractions from sucrose gradients as in figure 4, from control (tracks 1, 2, 3 and 4 respectively) and from heat shocked cells (tracks 9, 10, 11 and 12 respectively), are seen respectively in the left and right panel. Translation reactions were for 60 min at 30 °C at rate limiting mRNA concentrations. Electrophoresis was run in a 15% polyacrylamide gel. Fluorographic exposure was for 5 h at −70 °C.

mass polypeptides were not resolved in this gel. In contrast to heat shock mRNA, the poly(A)$^+$ control mRNA from the gradient fraction equivalent to 20*S* directs the synthesis of a series of polypeptides many of which migrate at the position of those labelled *in vivo* (figure 4*c*). In this case there is little synthesis of 70000 molecular mass polypeptides, but a significant synthesis of polypeptides comigrating with the 84000 and 68000 molecular mass heat shock polypeptides. Poly(A)$^+$ control mRNA from the gradient fraction equivalent to 12*S* directs the synthesis of a number of low molecular mass polypeptides, whose pattern in distinctly different from that seen in the case of heat shock mRNA (figure 4*d*). We cannot exclude a low synthesis of the small heat shock polypeptides. Poly(A)$^+$ mRNA from two gradient fractions (15 and 18*S*), sedimenting between the heat shock 20 and 12*S* mRNA fractions (figure 4*b*) directs the

synthesis of small amounts of polypeptides, most of which comigrate with the predominant translation products made in cells at 25 °C, in particular a strong band at around 42000 molecular mass.

Heat shock mRNAs fractionated by electrophoresis on polyacrylamide gels (see preceding section) were assayed *in vitro* for protein synthesis and the results are shown in figures 9 and 10. The most abundant heat shock mRNA (fraction II) directs the synthesis of a 70000 polypeptide plus small amounts of larger products (figure 9). A 68000 molecular mass polypeptide was synthesized *in vitro* by a minor mRNA species (fractions III and IIb) migrating slightly faster

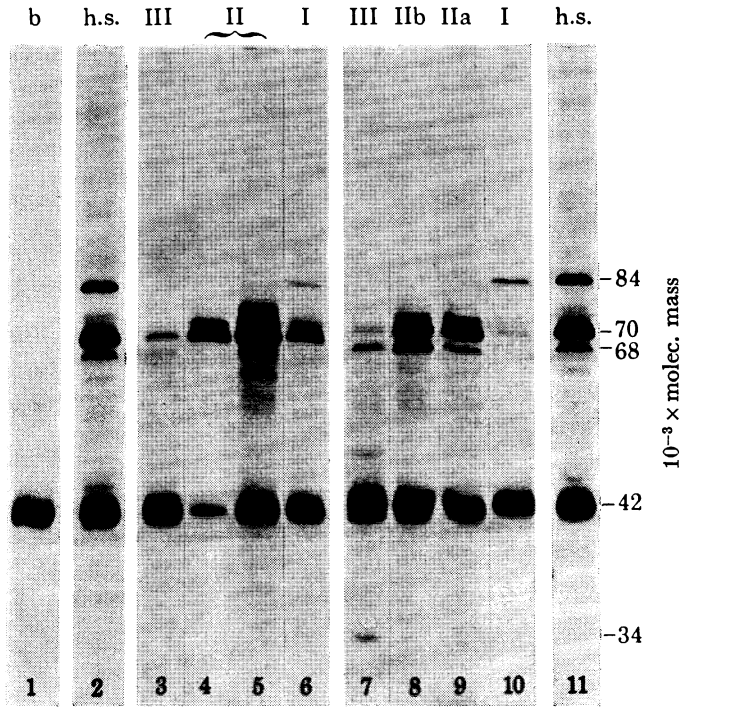

FIGURE 9. Fluorogram of *in vitro* translation products of 20*S* heat shock mRNAs fractionated by gel electrophoresis as in figure 5. The first track shows the blank, the *in vitro* system without the addition of mRNA. The strong band at around 42000 molecular mass is due to *in vitro* labelling of a protein independent of added mRNA, which is only seen after long exposure (Pelham & Jackson 1976). In tracks 2 and 11, *in vivo* labelled heat shock polypeptides, mixed with the blank, are shown as references. The polypeptides synthesized *in vitro* by the poly(A)+ heat shock mRNA fractions III, II and I from *large* polyribosomes (see figure 5*a*) are seen in tracks 3, 5 and 6 respectively. Track 4 shows a shorter exposure of track 5. The polypeptides synthesized *in vitro* by the poly(A)+ heat shock mRNA fractions III, IIb, IIa and I from *small* polyribosomes (see figure 5*b*) are shown in track 7, 8, 9 and 10 respectively. The reactions were carried out at 30 °C for 60 min. Estimated from the specific activity, the amounts of RNA added in tracks 3–10 were roughly 25, 215, 42, 35, 103, 101 and 30 ng respectively. Electrophoresis was run in a 15% polyacrylamide gel with 0.086% *bis*-acrylamide for 22 h at 120 V. Fluorographic exposure was for 12 days.

than the predominant heat shock mRNA, and still contaminated by the latter. The fingerprints of the 70000 and 68000 molecular mass polypeptides synthesized *in vitro* were identical to those of the heat shock polypeptides made *in vivo*. The minor bands just above the 70000 molecular mass band gave similar fingerprints to that of the 70000 polypeptide. In addition to a 68000 band, mRNA from fraction III (figure 5) also gives rise to lower molecular mass bands (40000 and 34000) the origin of which is not clear. Messenger RNA from fraction I directs the synthesis of an 84000 molecular mass polypeptide and also of some 70000 polypep-

tide, the latter probably due to trailing of fraction II RNA in the gel. The minor heat shock mRNA species in the gel are obviously still contaminated by variable amounts of the main heat shock mRNA. The strong band around 42000 seen throughout figure 9 is due to *in vitro* labelling of a protein independently of mRNA addition (Pelham & Jackson 1976). This band appears only after long exposure which are necessary with very low incorporation, close to the limit of detection.

Translation products of the 12*S* heat shock mRNA fractions, as seen in figure 6, were analysed and the results are shown in figure 10. Only partial resolution of at least three mRNA species has been achieved so far in this way.

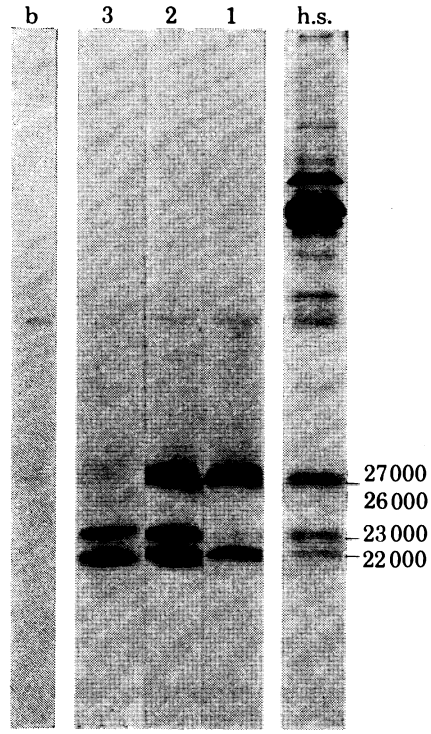

FIGURE 10. Fluorogram of *in vitro* translation products of 12*S* heat shock mRNAs fractionated by gel electrophoresis as in figure 6. The first track shows the blank, the *in vitro* reaction mixture with no RNA added. Tracks 2, 3 and 4 show the polypeptides synthesized *in vitro* by heat shock mRNA fractions 3, 2 and 1 respectively as shown in figure 6. Track 5 shows as a reference *in vivo* labelled heat shock polypeptides (as in figure 8). The translation reaction was run for 60 min at 30 °C. The [^{35}S]methionine labelled products were analysed by electrophoresis on a 15% polyacrylamide gel with 0.086% *bis*-acrylamide. Fluorographic exposure was for 5 h at −70 °C.

DISCUSSION

The heat shock induced in *Drosophila melanogaster* a series of 9–10 puffs (Ritossa 1962; Ashburner 1970) and the synthesis of a number of messenger RNA species which code altogether for about eight polypeptides all shown here to have distinctly different tryptic fingerprints. RNA synthesized in tissue culture cells during a heat shock hybridizes *in situ* on the giant chromosomes from salivary glands at sites corresponding to heat shock puffs (McKenzie *et al.* 1975; Spradling *et al.* 1975). Using purified messenger RNAs for both hybridization *in situ* and protein synthesis *in vitro*, it can be expected that a precise correlation between puffs and proteins will soon be established.

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For the purification of messenger RNA, it is essential to use an assay to test for specific messengers. The cell-free system for protein synthesis described recently by Pelham & Jackson (1976) was found to be suitable. In this system the evidence obtained indicates that heat shock messenger RNA preparations directed the synthesis of most, if not all, heat shock polypeptides with virtually no by-product. The labelled polypeptides made *in vitro* separated by SDS–polyacrylamide gel electrophoresis can be fingerprinted and compared with those made *in vivo*. This was done so far for the 70000 and 68000 molecular mass polypeptides and the fingerprints of the products made *in vivo* and *in vitro* were found to be identical. It is interesting that when 20*S* crude heat shock messenger RNA from sucrose gradients was translated *in vitro*, there was a striking correspondence in the relative amount of the three heat shock polypeptides, at molecular masses 84000, 70000 and 68000, synthesized *in vivo* and *in vitro*.

We have shown that the main heat shock RNA (molecular mass *ca.* 0.9×10^6) unambiguously codes for the 70000 molecular mass heat shock polypeptide, and an apparently larger RNA species codes for the 84000 band. The identification of the messenger RNA for the 68000 molecular mass polypeptide still remains uncertain since the RNA fractions tested directed the synthesis of the 68000 polypeptide plus additional polypeptides at about 40000 and 34000. The partial resolution of the 12*S* heat shock RNA allows us to conclude that it consists of at least three different messenger species, more probably four, coding for the 26000–27000, 23000 and 22000 molecular mass heat shock polypeptides. Therefore our results provide evidence for the existence of at least six distinct heat shock messenger RNA species, coding each for a distinct polypeptide. It would not be surprising if a one-to-one correlation might hold for the whole series of heat shock polypeptides.

A tentative correlation between a polypeptide and a heat shock puff locus can be drawn with the data available now: we have seen that the major 20*S* heat shock messenger RNA codes for the 70000 molecular mass polypeptide, and McKenzie *et al.* (1975) have reported that a similar RNA fraction hybridizes *in situ* mainly at the heat shock puff loci 87 B/C and 87 A.

The question of the rôle of the heat shock proteins is totally open. The synthesis of these proteins appears to be induced as a physiological response to a variety of conditions leading to a state of shock such as high temperature, ammonia, uncoupling of oxidative phosphorylation and recovery from anaerobiosis. These proteins might thus have important functions. At this point, however, it cannot be excluded that the heat shock genes are activated at the higher temperature by a non-physiological effect.

We wish to thank Dr R. Jackson for introducing one of us (M.-E.M.) to the use of the reticulocyte system for protein synthesis developed in his laboratory, Dr B. Allet for advice on the fingerprinting techniques, Dr G. Spohr for an electrophoretic analysis in formamide gels, Dr J.-L. Darlix for a sample of ribonuclease H, Dr G. Echalier for a strain of *Drosophila melanogaster* tissue culture cells, Miss J. Rosselet and Miss F. Vauclair for excellent technical assistance and Mr O. Jenni for drawings and plates.

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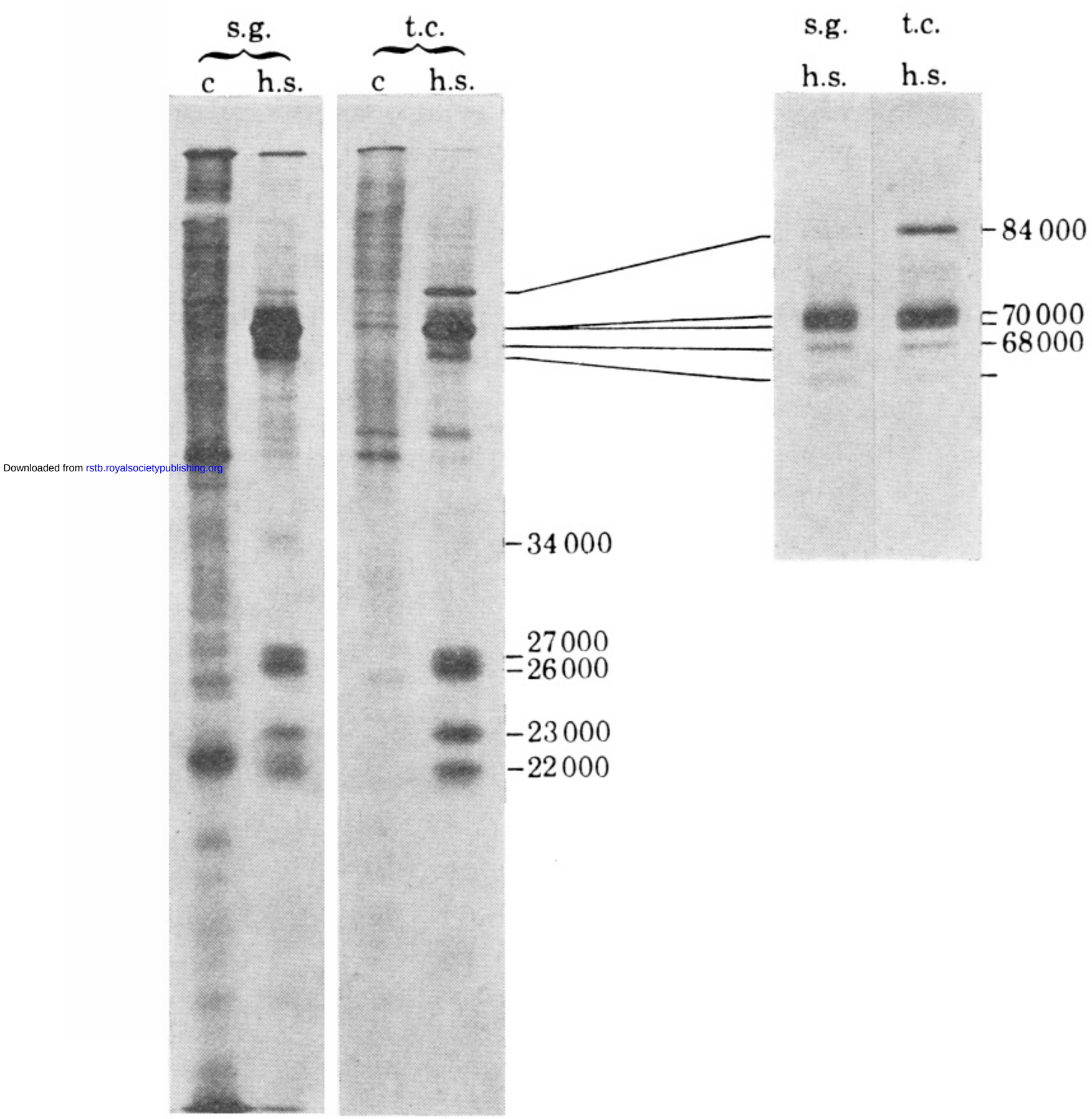

FIGURE 1. The effect of heat shock on the gel electrophoresis autoradiograph pattern of [^{35}S]methionine labelled proteins from tissue culture cells and salivary glands. Tissue culture cells (t.c.) were labelled for 1 h at 37 °C following a heat shock (h.s.) of 2 h at the same temperature. Control (c) cells were labelled in a parallel incubation at 25 °C. Salivary glands (s.g.) were labelled after heat shock as previously described (Tissières *et al.* 1974). Control (c) glands were labelled in a parallel incubation at 25 °C. The proteins were separated by SDS–polyacrylamide gel electrophoresis and detected by autoradiography of the dried gels. The concentrations of the gels and the conditions of electrophoresis were: at the left, 12.5% acrylamide, 0.33% *bis*-acrylamide, and 50 V for 17 h; at the right, 15.0% acrylamide, 0.09% *bis*-acrylamide, and 130 V for 17 h. The apparent molecular masses were determined as indicated in Materials and Methods.

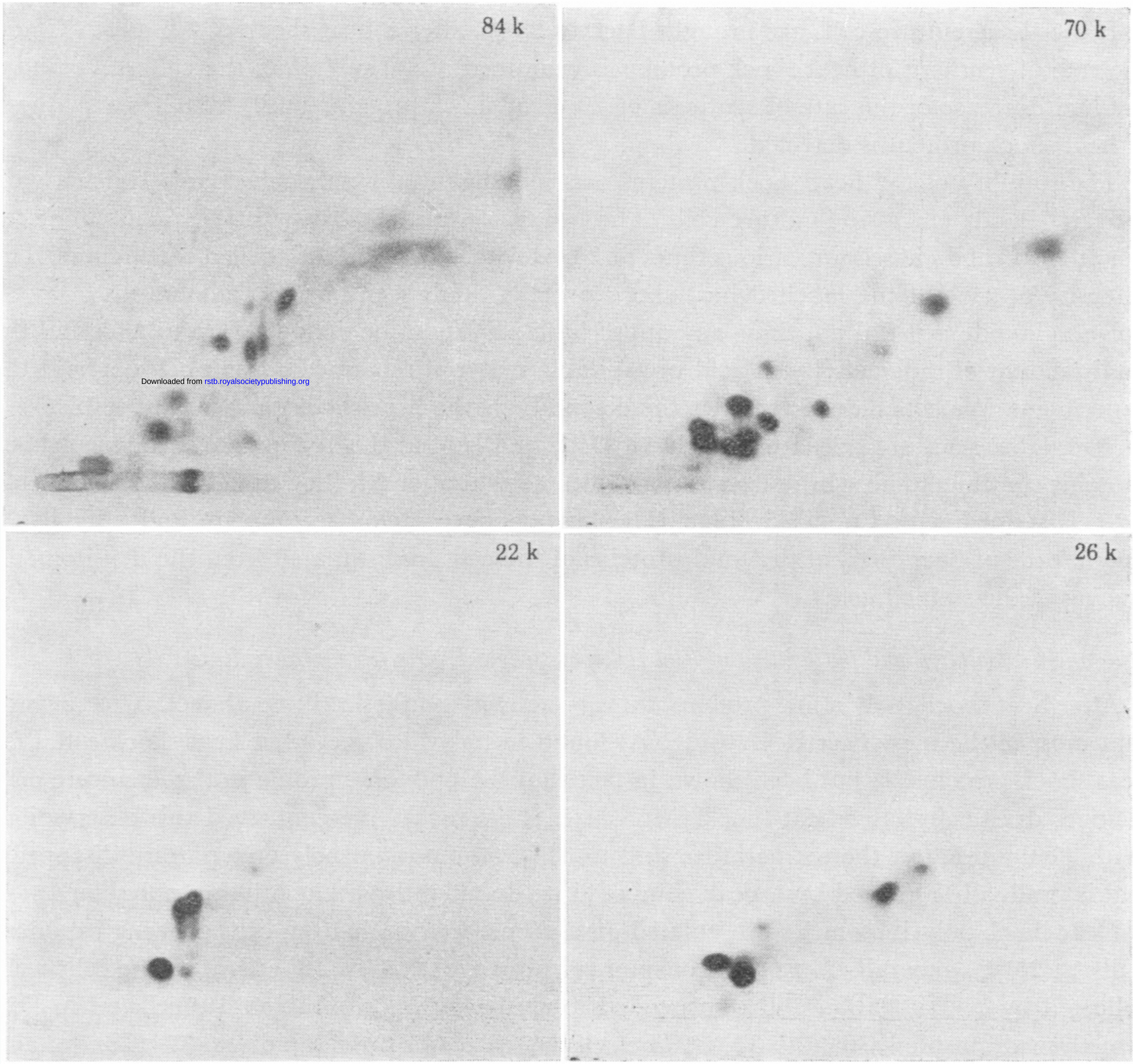

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FIGURE 2. Tryptic fingerprints of the 84000, 70000, 26000 and 22000 molecular mass heat shock polypeptides from tissue culture cells labelled *in vivo* with [^{35}S]methionine.

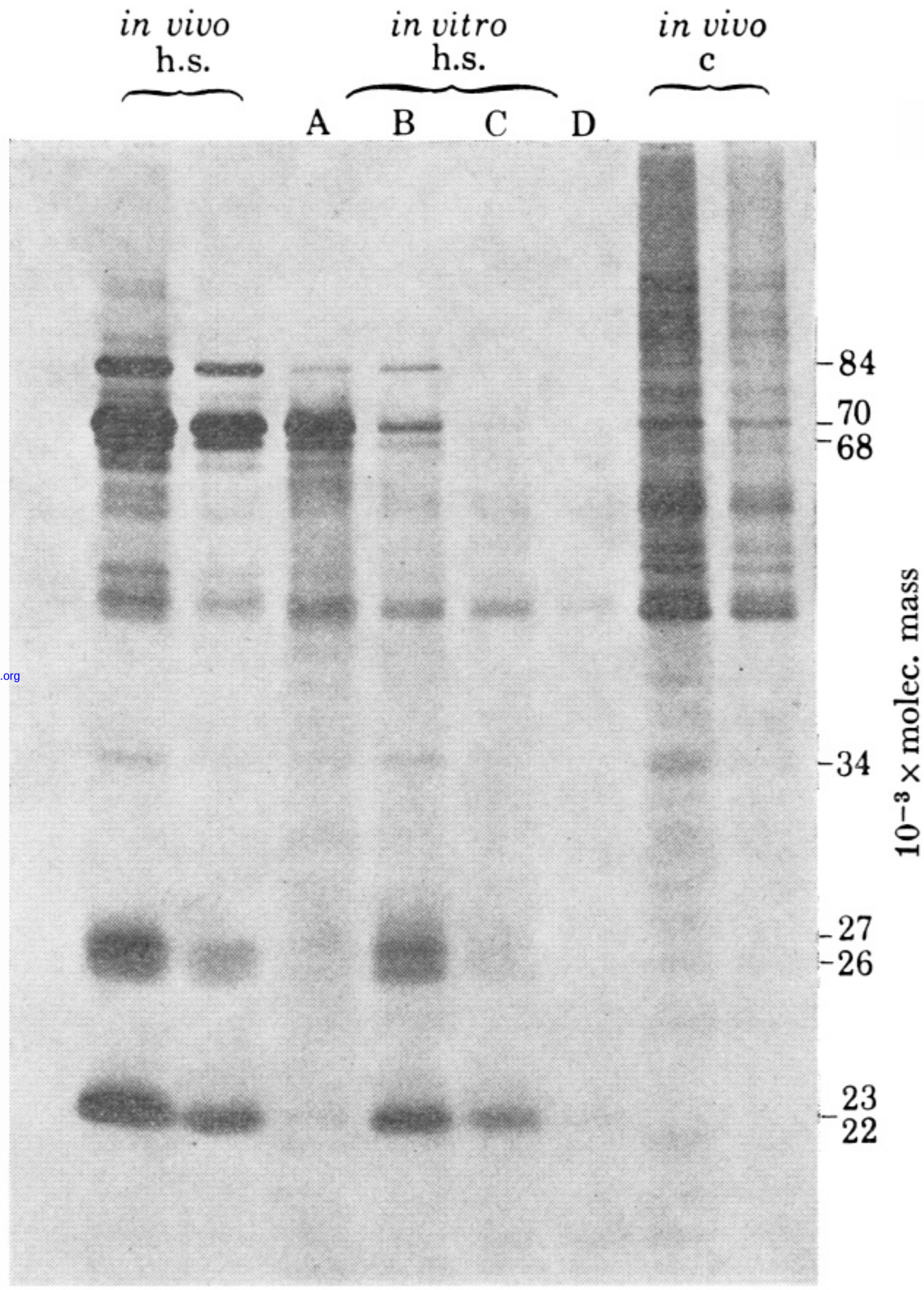

FIGURE 7. Translation of poly(A)$^+$ messenger RNA from polysomal fractions (see figure 3). Fluorogram of [^{35}S]methionine labelled polypeptides synthesized *in vitro* and *in vivo*, separated by electrophoresis in SDS–polyacrylamide gels. *In vivo* labelled heat shock polypeptides are seen in the second and third tracks at two different concentrations (ratio 5:2). *In vivo* labelled control polypeptides (at 25 °C) are seen in the eighth and ninth tracks at two different concentrations (ratio 5:2). Polypeptides synthesized *in vitro* by poly(A)$^+$ heat shock mRNA from fractions A, B, C and D (figure 3) are seen in the centre of the figure. Translation reactions were run for 20 min at 30 °C with 0.57, 0.7, 0.71 and 1.1 μg of RNA corresponding respectively to 0.25% of each fraction A, B, C and D. The translation product labelled *in vitro* in the absence of RNA (blank) is seen at the left in the figure. The reaction mixtures (see Methods) were diluted with electrophoresis sample buffer to 120 μl. Analysis by electrophoresis of 3 μl of each sample was carried out in a 15% polyacrylamide gel with 0.2% *bis*-acrylamide (see Methods). Fluorographic exposure was for 3 days at −70 °C.

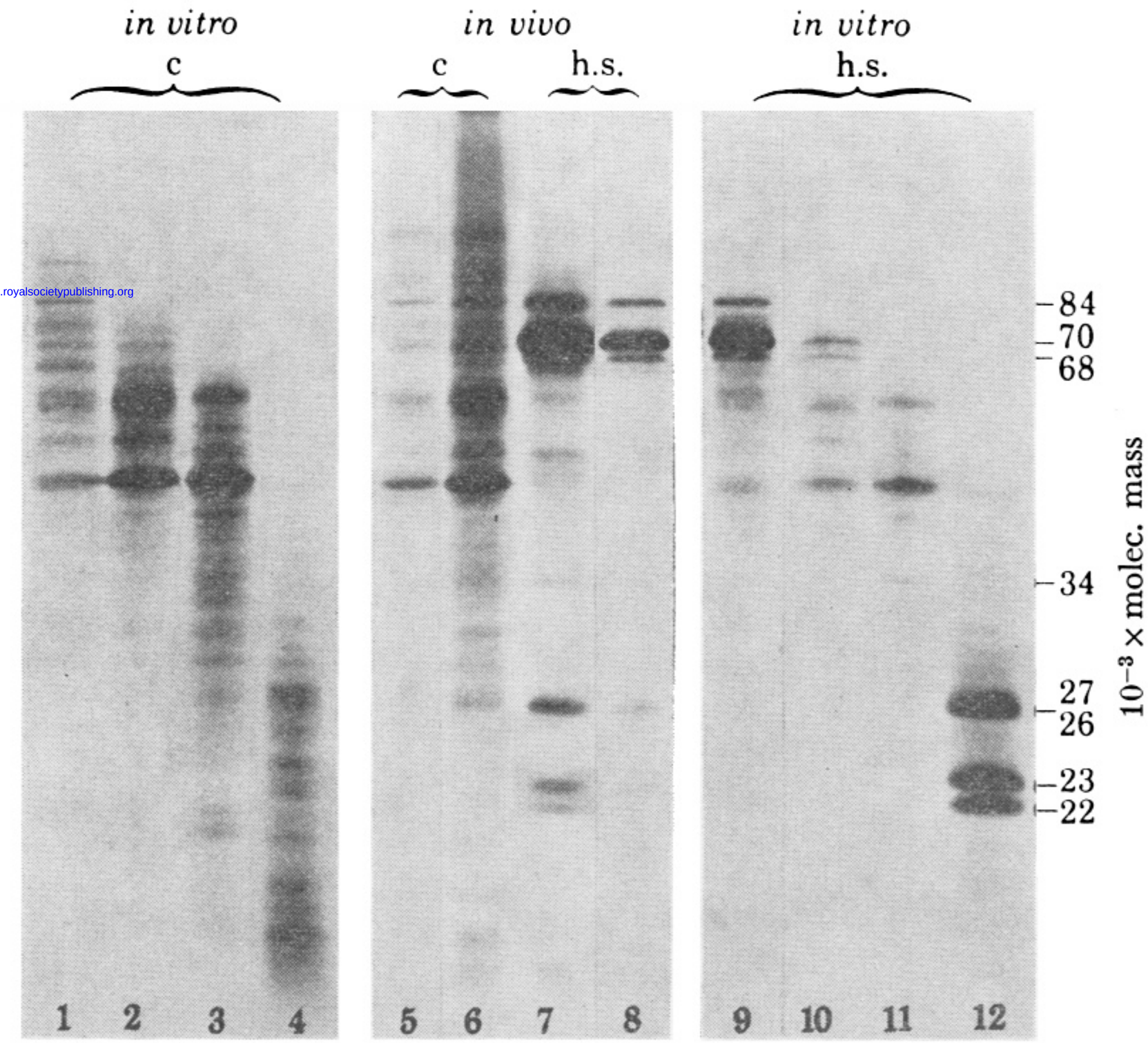

FIGURE 8. Fluorogram of [^{35}S]methionine labelled polypeptides synthesized *in vitro* by poly(A)$^+$ mRNA fractions from sucrose gradients (as in figure 4). In the centre panel, the polypeptides labelled for 1 h *in vivo* in control cells, at 25 °C (tracks 5 and 6) or in cells heat shocked for 1 h at 37 °C (tracks 7 and 8) are seen at two different exposures (5 h and 19 h). Polypeptides synthesized *in vitro* by 20, 18, 15 and 12*S* poly(A)$^+$ mRNA fractions from sucrose gradients as in figure 4, from control (tracks 1, 2, 3 and 4 respectively) and from heat shocked cells (tracks 9, 10, 11 and 12 respectively), are seen respectively in the left and right panel. Translation reactions were for 60 min at 30 °C at rate limiting mRNA concentrations. Electrophoresis was run in a 15% polyacrylamide gel. Fluorographic exposure was for 5 h at −70 °C.

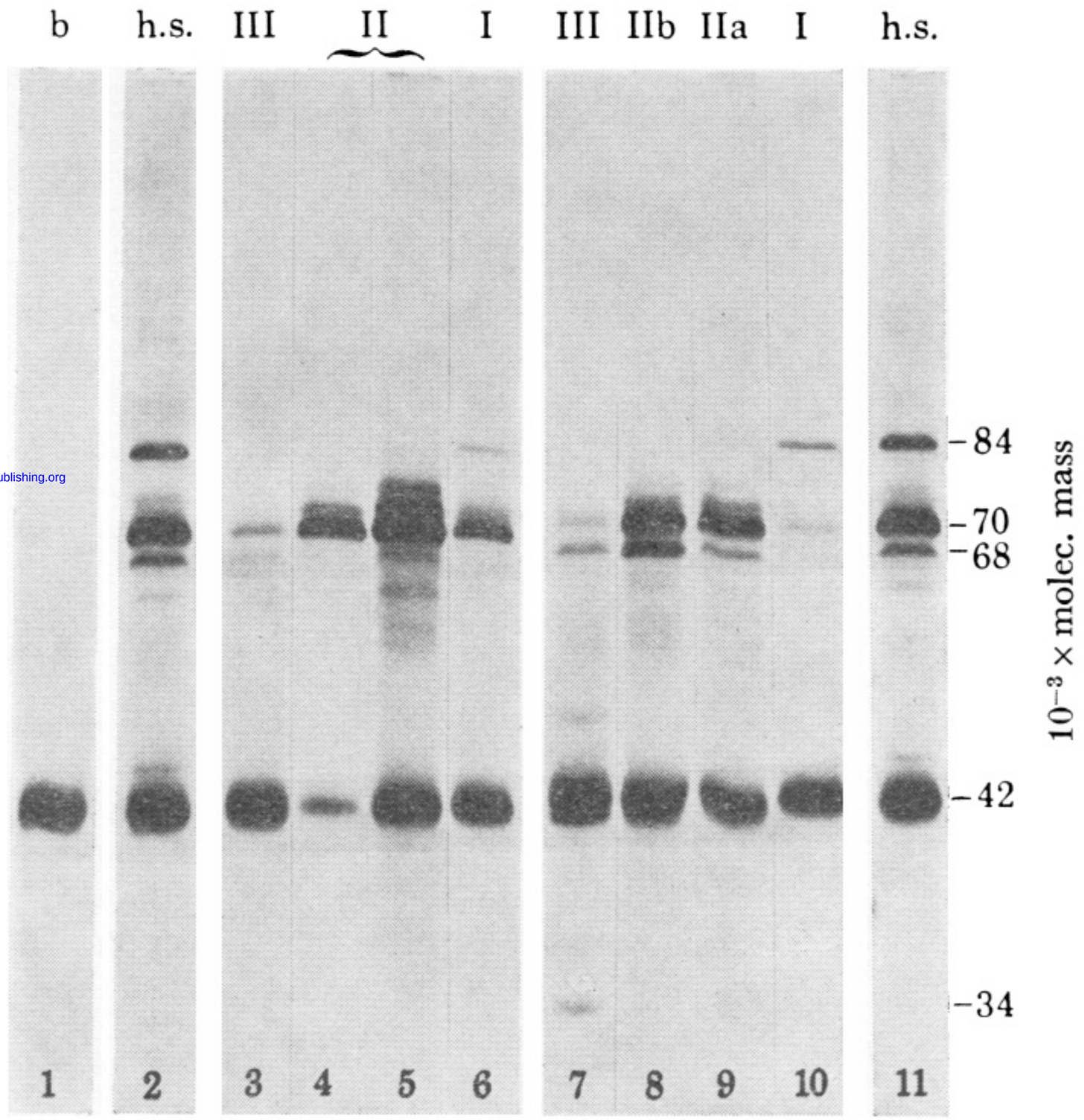

FIGURE 9. Fluorogram of *in vitro* translation products of 20*S* heat shock mRNAs fractionated by gel electrophoresis as in figure 5. The first track shows the blank, the *in vitro* system without the addition of mRNA. The strong band at around 42000 molecular mass is due to *in vitro* labelling of a protein independent of added mRNA, which is only seen after long exposure (Pelham & Jackson 1976). In tracks 2 and 11, *in vivo* labelled heat shock polypeptides, mixed with the blank, are shown as references. The polypeptides synthesized *in vitro* by the poly(A)$^+$ heat shock mRNA fractions III, II and I from *large* polyribosomes (see figure 5*a*) are seen in tracks 3, 5 and 6 respectively. Track 4 shows a shorter exposure of track 5. The polypeptides synthesized *in vitro* by the poly(A)$^+$ heat shock mRNA fractions III, IIb, IIa and I from *small* polyribosomes (see figure 5*b*) are shown in track 7, 8, 9 and 10 respectively. The reactions were carried out at 30 °C for 60 min. Estimated from the specific activity, the amounts of RNA added in tracks 3–10 were roughly 25, 215, 42, 35, 103, 101 and 30 ng respectively. Electrophoresis was run in a 15% polyacrylamide gel with 0.086% *bis*-acrylamide for 22 h at 120 V. Fluorographic exposure was for 12 days.

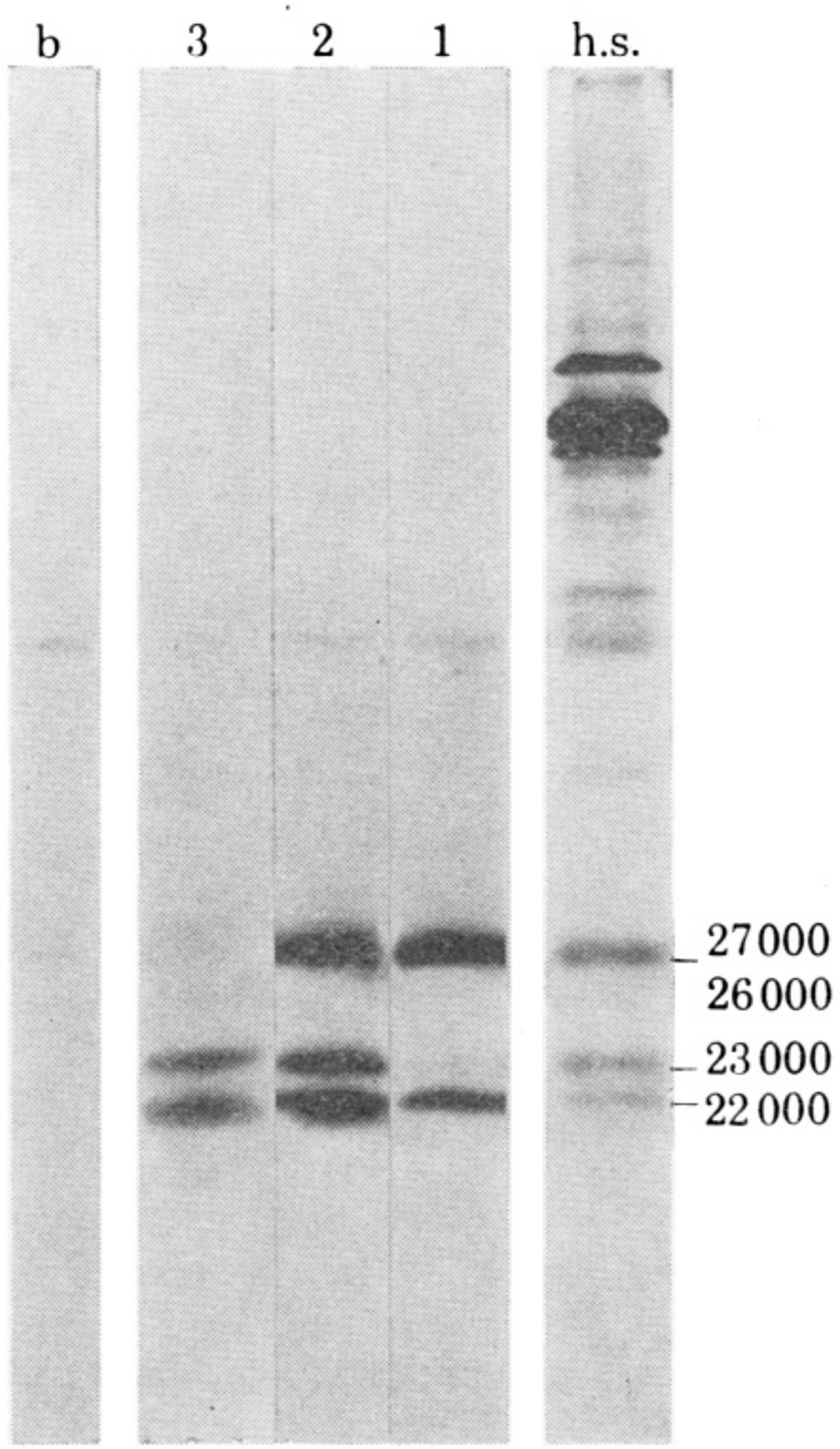

FIGURE 10. Fluorogram of *in vitro* translation products of 12*S* heat shock mRNAs fractionated by gel electrophoresis as in figure 6. The first track shows the blank, the *in vitro* reaction mixture with no RNA added. Tracks 2, 3 and 4 show the polypeptides synthesized *in vitro* by heat shock mRNA fractions 3, 2 and 1 respectively as shown in figure 6. Track 5 shows as a reference *in vivo* labelled heat shock polypeptides (as in figure 8). The translation reaction was run for 60 min at 30 °C. The [^{35}S]methionine labelled products were analysed by electrophoresis on a 15% polyacrylamide gel with 0.086% *bis*-acrylamide. Fluorographic exposure was for 5 h at −70 °C.