The major mammalian heat shock or "stress" proteins (molecular masses of 90,000, 72,000, and 73,000 daltons) have been purified from stressed HeLa cells. The 90,000-dalton protein co-purified with small amounts of a 100,000-dalton protein which was identified as one of the other stress proteins in these cells. The 72,000- and 73,000-dalton proteins co-purified throughout the fractionation scheme, apparently as a mixture of monomeric forms of the two proteins. From sedimentation velocity and gel filtration analysis, it was found that the 90,000/100,000-dalton protein mixture had a Stokes radius of 68 Å and a $\phi_{2n}$ value of 5.8 while the 72,000/73,000-dalton protein mixture had a Stokes radius of 42.6 Å and a $\phi_{2n}$ value of 4.3. The purified proteins migrated identically in two-dimensional gel electrophoregrams with their counterparts from total cell lysates of $[^{35}S]$methionine-labeled stressed HeLa cells. Peptide mapping experiments indicated that the 72,000- and 73,000-dalton proteins contained common peptides while the 90,000- and 100,000-dalton proteins appeared to be distinct. Amino acid analysis of the 90,000- and a mixture of the 72,000/73,000-dalton proteins showed that both contained relatively high amounts of Asp/Asn and Glu/Gln.

Although it is known which proteins are induced during the stress response, their location and function in the cell have not been fully elucidated. There is evidence that induction of the stress response confers a degree of protection against subsequent stress situations and that such a protection is contingent upon the prior synthesis of the stress proteins (18-20). Furthermore, with regard to their intracellular localization, there are reports showing that in insect cells some of the stress proteins appear to migrate to the nucleus shortly after their synthesis in the cytoplasm (21-25).

As a first step in the analysis of the function and intracellular location of the mammalian stress proteins, we report here the purification of three of the six stress proteins produced in HeLa cells. This purification scheme has been subsequently employed in the purification of the corresponding proteins present in normal unstressed cells. Acquisition of both the purified proteins (as well as the appropriate antibodies directed against them) should aid in the determination of the location and function(s) of the stress proteins both in normal and in stressed cells and thereby facilitate and expedite a more complete understanding of the stress response.

**EXPERIMENTAL PROCEDURES**

**Growth of Cells and Induction of the Stress Response.** Two liters of HeLa cells, seeded at 5 x 10^5 cells/ml, were grown in suspension in F-13 spinner medium (Gibco) supplemented with 5% horse serum. The stress response was initiated 24 h later by one of three methods: (a) addition of L-azetidine-2-carboxylic acid (Calbiochem) (final concentration, 5 mM); (b) addition of ZnCl₂ (final concentration, 0.25 mM); or (c) heating the medium to 42 °C. Between 8 and 16 h later (the time depending upon the treatment used for the induction), the cells were harvested and prepared for protein purification as described under "Results."

**Column Chromatography and Buffers.** Whatman DEAE-cellulose (DE22 and DE52) was purchased from Beeve Angel and Co. Sepharose 6B-CL and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals. Hydroxylapatite was purchased from Bio-Rad. Salt

*The abbreviations used are: SDS, sodium dodecyl sulfate; AzC: L-azetidine-2-carboxylic acid; DME, Dulbecco's modified Eagle's medium; 72 kd, 73 kd proteins, etc., proteins of 72 and 73 kilodaltons.
concentrations in column effluents were measured with the use of a Radiometer conductivity meter. Buffer b (see below) gave a reading of 1.9 milliseemens. Absorbance at 280 nm of the column effluents was measured with a Varian 634 spectrophotometer.

Standards used to prepare a calibration curve for molecular weight determination by gel filtration were purchased from Pharmacia Fine Chemicals and included blue dextran (MW = 2,000,000), ovalbumin (MW = 43,000 and Stokes radius of 30.5Å), and catalase (MW = 240,000 and Stokes radius of 52.2Å).

Buffers used through the purification were designated Buffer b and Buffer c. Buffer b contained 20 mM Tris-acetate, pH 7.6, 20 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol. Buffer c contained 20 mM sodium phosphate, pH 7.6, 0.1 mM EDTA, 15 mM 2-mercaptoethanol.

**SDS-Polyacrylamide Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis was performed essentially as described by Blattler et al. (29) except that the buffer system of Laemmli (27) was used. Protein samples were solubilized in electrophoresis sample buffer at a final concentration of 1% SDS, 50 mM of dithiothreitol, 40 mM of Tris-HCl, pH 6.8, 7.5% glycerol, and bromophenol blue. Molecular mass markers, purchased from Bio-Rad Laboratories, included myosin (200,000 daltons), β-galactosidase (130,000 daltons), phosphorylase b (94,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), soybean trypsin inhibitor (21,000 daltons), and lysozyme (14,000 daltons). Gels were stained with 1.5% Coomassie brilliant blue in 45% methanol, 10% acetic acid and destained in 12% methanol, 12% acetic acid.

**Sucrose Density Gradient Centrifugation**—Centrifugation of proteins through sucrose was done according to the procedure of Martin and Ames (28). Protein standards were purchased from Pharmacia Fine Chemicals and included chymotrypsinogen (s~20w~ = 2.6) and aldolase (s~20w~ = 7.4). The 5-20% (w/v) sucrose gradients were centrifuged in a SW 50.1 Ti rotor (Beckman Instruments) at 38,000 rpm for 15 to 25 h at 4 °C in a L5-65 Beckman preparative ultracentrifuge.

**Two-dimensional Isoelectric Focusing and SDS-Polyacrylamide Gel Electrophoresis**—The analysis was done as described by Garrels (29). Gels were stained with Coomassie blue as described above. Fluorography was done as described by Garrels (29).

**[35S]Methionine-labeling of Normal and Stressed Cells**—HeLa cells (~1 x 10⁶ cells/dish) on 35-mm plastic dishes (Falcon) were grown in DMEM containing 2% calf serum. Cells were stressed by the addition of 5 mM AzC or 0.25 mM ZnCl₂ to the medium, or by growth in normal medium at 42 °C. For labeling, the medium was removed, the cells were washed with DMEM lacking methionine, and then were labeled in methionine-free DMEM supplemented with [35S]methionine and 2% dialyzed calf serum under the appropriate stress condition. Following the labeling period, the medium was removed, the cells

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**Fig. 1. Polypeptide composition of normal and stressed HeLa cells.** HeLa cells, on 35-mm Falcon dishes, were grown in DMEM supplemented with 2% calf serum (lane B) or in DME supplemented with 2% calf serum and 5 mM AzC (lane C) for 16 h. The cells were washed twice with phosphate-buffered saline and then solubilized by the addition of SDS-gel electrophoresis sample buffer. The polypeptides were separated on a 10% polyacrylamide gel and stained with a 10% gel as described above. Shown in lanes D-F is an autoradiograph of the gel. Molecular mass markers, indicated on the left, in descending order are: myosin (200,000), β-galactosidase (130,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), and carbonic anhydrase (30,000). Lane A, molecular mass markers stained with Coomassie blue. Lane B, Coomassie blue-stained polypeptides present in normal HeLa cells. Lane C, Coomassie blue-stained polypeptides present in HeLa cells incubated in 5 mM AzC for 16 h. Lane D, [35S]methionine pulse-labeled polypeptides synthesized in normal HeLa cells. Lane E, [35S]methionine pulse-labeled polypeptides synthesized in HeLa cells incubated in 5 mM AzC for 16 h. Lane F, [35S]methionine pulse-labeled polypeptides synthesized in HeLa cells incubated in 5 mM AzC for 24 h.

**Fig. 2. Fractionation of heat-shocked HeLa cells.** HeLa cells, grown in suspension culture, were incubated at 42 °C for 6 h in F-13 spinners medium supplemented with 5% horse serum. The cells were harvested, washed several times with phosphate-buffered saline, and then quickly washed with hypotonic medium (20 mM Tris-acetate (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol). After collection by centrifugation, the cells were lysed by Dounce homogenization in cold hypotonic lysis buffer (10 mM Tris-acetate (pH 7.5), 10 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride). The cell lysate was adjusted to 0.25 M sucrose, 1 mM MgCl₂ and centrifuged at 1,000 x g (average) for 10 min at 4 °C. The 1,000 x g supernatant was then centrifuged at 100,000 x g (average) for 1 h at 4 °C. Equal fractions of the pellets and supernatants were then analyzed on a 10% SDS-polyacrylamide gel. Molecular mass markers (lane A) were, in descending order: 200,000, 130,000, 94,000, 68,000, 43,000, 30,000, and 21,000 daltons. The 100, 90, 80, 73, and 72 kd stress proteins are indicated by hash marks to the left. Lane B, Dounce-homogenized cell lysate (0.015% of total). Lane C, 1,000 x g pellet (0.03% of total). Lane D, 1,000 x g supernatant (0.03% of total). Lane E, 100,000 x g supernatant (0.06% of total). Lane F, 100,000 x g pellet (0.06% of total).
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3. A280

I I I I

cond.

0 10 20 30 40 50 60 70

Fraction no.

FIG. 3. DE52-cellulose column chromatography. HeLa cells were incubated in F-13 spinners medium containing 5% horse serum, 5 mM AzC and harvested after 16 h. The cells were washed with phosphate-buffered saline and swollen in hypotonic medium. Swollen cells were broken open by Dounce homogenization, and the soluble proteins were separated from insoluble material by centrifugation at 20,000 × g for 15 min at 4 °C. The supernatant was applied to a DE52-cellulose column (1.2 × 20 cm) equilibrated in Buffer b. The elution profile for the column developed with a linear gradient of 20 to 500 mM NaCl in Buffer b (180 ml total) is shown in the upper portion. The flow rate was 25 ml/h. Fractions of 2.2 ml were collected and analyzed for their A280 (O) and conductivity (millisem) (□). A 10% SDS-polyacrylamide slab gel of the starting material (lane 2), the material applied to the column following the 20,000 × g centrifugation (lane 4), the material which flowed directly through the column (lane 5), and every other effluent fraction (the beginning of the gradient is indicated by the arrow) are shown in the lower portion. For reference, the polypeptides present in cells treated with 5 mM AzC for 16 h are shown in lane 3. Proteins were stained with Coomassie blue, and molecular mass markers (far left and far right lanes) were the same as described in Fig. 2.

The proteins were then electrophoresed from the gel slices as described by Welch et al. (30), dialyzed extensively against 30 mM NH4HCO3, 0.01% SDS, and lyophilized. The lyophilized proteins were then resuspended in 10% acetic acid and passed over a G-25 column to remove all salts and any residual glycine from the SDS-gel electrophoresis running buffer. Fractions from the G-25 column were lyophilized, oxidized with performic acid, and hydrolyzed in 6 N HCl at 100 °C for 24 h in vacuo. Amino acid compositions were determined using a Beckman 119CL automated analyzer.

Iodination of the Individual Stress Proteins and One-dimensional
Peptide Maps—The purified 72/73 kd mixture obtained after gel filtration over Sepharose 6B-CL and the purified 90/100 kd mixture obtained after gel filtration over Sephacryl S-300 were solubilized by the addition of SDS-gel electrophoresis sample buffer, and the proteins were chromatographed on a 7.5% SDS-polyacrylamide gel, and visualized by Coomassie blue staining. Individual 72, 73, 90, and 100 kd proteins were carefully excised from the gels, electroeluted out of the gel slices, dialyzed extensively against 20 mM sodium phosphate (pH 7.4), 0.01% SDS, and lyophilized. The individual proteins were resuspended in H2O and iodinated with Na125I using the chloramine-T method (31). Following their iodination, the proteins were passed over a G-25 column, and the peak fractions were combined, solubilized in SDS-gel electrophoresis sample buffer, and chromatographed on a 10% SDS-polyacrylamide gel. The gel was dried and the protein was visualized by autoradiography.

For peptide mapping, the individual proteins were excised from the dried gel and mapped essentially as described by Cleveland et al. (32).

RESULTS
The purification procedures described below have been applied to cells grown in normal medium and cells stressed by either growth in medium containing 5 mM AzC, growth in medium containing 0.25 mM ZnCl2, or growth at 42°C in normal medium. In general, except for the absolute amounts of the stress proteins induced by these various treatments, essentially identical results were obtained with respect to the purification, gel filtration, and sedimentation characteristics of the proteins. However, for the sake of brevity, the results of the fractionation of HeLa cells stressed by growth in the presence of AzC are presented.

The addition of AzC to the growth medium results in the elevated synthesis of a small number of polypeptides concomitant with the decreased synthesis of most other cellular polypeptides (Fig. 1; see also Ref. 8 for a two-dimensional gel analysis of the response). While Coomassie blue staining of the proteins present in normal HeLa cells (lane B) and in HeLa cells exposed to AzC for 16 h (lane C) shows the accumulation of perhaps two polypeptides (i.e., Mr = 80,000 and 72,000), brief labeling of the cells with [35S]methionine shows the elevated synthesis of at least six polypeptides with
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$M_r = 110,000, 100,000, 90,000, 80,000, 73,000, \text{ and } 72,000$ (lanes D and E). (The elevation of the rate of synthesis of these six polypeptides is a reproducible affect of stress while the elevation of the rate of synthesis of several other polypeptides (e.g. 60 and 40 kDa) is variable; thus, until further experimental results warrant a change, the six aforementioned polypeptides constitute the “stress proteins” in these cells.) Treatment with AzC for 24 h or longer, however, resulted in the death of most of the cells, with the residual cells synthesizing essentially only the stress proteins (lane F). Heat shock treatment of HeLa cells (i.e. 42 °C) for 8 h or growth of the cells in the presence of 0.25 mM ZnCl₂ resulted in a similar induction of the six major stress proteins described above (data not shown).

Initial Steps in the Purification of the Stress Proteins—In order to facilitate the purification of the stress proteins, we first examined their relative subcellular distribution, using a simple fractionation procedure based on velocity sedimentation. Following their incubation at 42 °C (i.e. heat shock) for 8 h, HeLa cells were collected by centrifugation and washed three times with cold phosphate-buffered saline. The cells were swollen by washing once in cold hypotonic medium (50 mM NaCl, 20 mM Tris-acetate (pH 7.5), 0.1 mM EDTA, 15 mM 2-mercaptoethanol) and after collection by centrifugation lysed by Dounce homogenization in cold hypotonic lysis buffer (10 mM Tris-acetate (pH 7.5), 10 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). MgCl₂ was added to a final concentration of 1 mM and the cell lysate was then fractionated by two velocity sedimentation centrifugations. The protein composition during each step of the fractionation was determined by SDS-gel electrophoresis (Fig. 2). The identification of the stress proteins in each of the crude fractions was confirmed by two-dimensional gel electrophoresis (not shown). The Dounce-homogenized cell lysate (Fig. 2, lane B) was adjusted to 0.25 M sucrose and centrifuged at 1000 × g

**Fig. 5.** Sephacryl S-300 column chromatography of 90 and 100 kDa proteins. Fractions eluted from the hydroxylapatite column (Fig. 4) and containing the bulk of 90 and 100 kDa were pooled, dialyzed, and concentrated against Buffer b. The concentrated solution (1.5 ml) was applied to a Sephacryl S-300 column (1.0 × 100 cm) equilibrated with and developed in Buffer b. The flow rate was 8 ml/h. 1.1-ml fractions (80 fractions total) were collected and their $A_{280}$ was determined (upper portion). Shown in the lower portion is a Coomassie blue-stained 10% SDS-slab gel of a portion of every third fraction.
(average) for 10 min at 4 °C. A large pellet was obtained and contained appreciable amounts of all six of the stress proteins (Fig. 2, lane C). In relative terms, little of the 90 and the 100 kd stress proteins were found in this low speed pellet. The supernatant, however, contained most of the 90 kd protein as well as considerable amounts of 72, 73, 80, and 100 kd proteins (lane D). The 1000 × g supernatant was then centrifuged at 100,000 × g (average) for 1 h at 4 °C and the protein composition of the supernatant and pellet was analyzed. The supernatant (lane F) and pellet (lane E) contained about equal amounts of both the 80 and 100 kd proteins while most of the 90, 72, and 73 kd proteins were found in the supernatant (lane F). Hence, the supernatant following a low speed centrifugation of the Dounce-homogenized cell lysate was used as described below for the purification of the 72, 73, and 90 kd proteins. (The low and high speed pellets, on the other hand, are used as the starting material for the purification of the 80 and 100 kd stress proteins.)

For the purification of the 90, 72, and 73 kd stress proteins, 2 liters of HeLa cells (~2 × 10^7 cells) were grown in suspension for 16 h in culture medium supplemented with 5% horse serum and 5 mM AEC. Suspensions were used to ensure adequate starting amounts of the stress proteins. The cells were harvested by centrifugation at 1000 × g for 15 min in a Sorvall SS-34 rotor. The cells were washed twice with cold phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} and once with cold hypotonic buffer (as above). The cellular pellet (approximatively 3–4 g of packed cells) was then resuspended in cold hypotonic lysis buffer (as above). After swelling on ice for 20 min, the cells were lysed by Dounce homogenization using a tight fitting pestle. The homogenate (analyzed by SDS-gel electrophoresis, Fig. 2, lane 2) was centrifuged at 20,000 × g (maximum) for 15 min in a Sorvall SS-34 rotor at 4 °C. A large pellet consisting of mitochondria, nuclei, and unbroken cells was obtained. The supernatant was separated from the pellet and diluted with Buffer b until the conductivity approached that of the conductivity of Buffer b. This solution (Fig. 3, lane 4) was applied to a column packed with DE52 (1.2 × 20 cm) equilibrated in Buffer b and the column subsequently was washed with buffer until the A_{280} returned to a base-line value. A number of polypeptides did not bind to the column (Fig. 3, lane 5). Those proteins which did bind to the column were eluted with a linear gradient of NaCl (Fig. 3; the beginning of the gradient is indicated by the arrow). Four principal peaks of optical density were found to elute from the column, two of which contained substantial amounts of three of the stress proteins. The first peak (fractions 20–34) was enriched in the 72 and 73 kd stress proteins, although both proteins were seen to extend well throughout the gradient. The third peak (fractions 35–45) contained substantial amounts of 90 kd and some 100 kd as well. It is important to note here that the 100 kd protein present in fractions 35–45 represents only a portion of the total present in the stressed cell. As described above, our subcellular fractionation results, as well as other recent studies, have shown that some of the 100 kd protein remains in the pellet following the 20,000 × g centrifugation of the Dounce-homogenized cell lysate (see "Discussion"). The two other stress proteins that are indicated in Fig. 1, the 110 and 80 kd proteins, were not unambiguously detected in this elution profile. As was shown in Fig. 2, a significant portion of the 80 kd protein remains with the pellet following the 1,000 × g centrifugation. For their further purification, the two peak fractions containing the 72 and 73 kd proteins and the 90 and 100 kd proteins were pooled separately and dialyzed extensively against Buffer c.

Purification of the 90 kd Stress Protein—The pooled fractions containing the majority of the 90 and 100 kd proteins were clarified by centrifugation at 10,000 × g for 10 min at 4 °C following dialysis against Buffer c. The supernatant was applied to a hydroxylapatite column (1.2 × 10 cm) equilibrated in Buffer c. The column was washed with buffer until the A_{280} returned to a base-line value and the proteins were eluted with a linear gradient of potassium phosphate (pH 7.5). One major peak of optical density was observed to elute from the column (Fig. 4). This peak, composed of fractions 45–56, contained the vast majority of the 90 kd protein. These fractions, containing most of the 90 kd protein and some of the 100 kd protein, were pooled and concentrated by negative pressure dialysis against Buffer b using a Micro-Pro-Dicon concentrator (Bio-Molecular Dynamics, Beaverton, OR).

The concentrated proteins, in a volume of approximately 1.5 ml, were chromatographed on a Sephacryl S-300 column (1.2 × 100 cm) developed in Buffer b. A single peak of optical density (fractions 42–49) eluted from the column (Fig. 5). The 90 kd protein, as well as some 100 kd, eluted together in these fractions with minimal contamination from other proteins. Further attempts to separate 90 and 100 kd proteins were unsuccessful. Approximately 8–10 mg of purified 90 kd protein was obtained from 3–4 g of cells (wet weight). This then represents the final step in the purification of the 90 kilodal-

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6 W. J. Welch and J. R. Feramisco, manuscript in preparation.
Purification of the 72 and 73 kd Stress Proteins—The pooled fractions from the DE52 column containing the 72 and 73 kd proteins were clarified after dialysis against Buffer c and applied to a hydroxylapatite column (1.2 × 10 cm) equilibrated in Buffer c. The column was washed with Buffer c until the $A_{280}$, revealed a base-line value. Proteins were then eluted with a linear gradient of potassium phosphate (pH 7.5) giving rise to two major and two minor peaks of optical density, the latter comprising the bulk of both the 72 and 73 kd proteins (Fig. 6). As in the initial step of the purification using DE52 chromatography, both the 72 and 73 kd proteins displayed a wide profile across the gradient. In addition, they again appeared to elute together in apparently an equimolar mixture. Fractions 54–70, containing the majority of the 72 and 73 kd proteins, were pooled and concentrated by negative pressure dialysis against Buffer b.

Once concentrated to a volume of approximately 2 ml, the solution containing the 72 and 73 kd proteins was applied to a Sephacryl S-300 column (1.2 × 100 cm) and the column was developed in Buffer b. As can be seen in Fig. 7, a number of proteins including 72 and 73 kd were included in the column. Those fractions containing most of the 72 and 73 kd proteins (fractions 35–45) were pooled conservatively to minimize contamination from the other polypeptides present.

The combined fractions containing 72 and 73 kd were then applied directly to a DE53 column (1.2 × 10 cm) equilibrated in Buffer b. Because of the slightly different performance characteristics of this resin compared to DE52, this step improved the purity of the 72 and 73 kd proteins. The proteins were eluted from the column with a linear gradient of NaCl (Fig. 8). A single peak of optical density containing most, if not all, of the 72 and 73 kd proteins was detected. Fractions 30–43, the peak fractions, were pooled and concentrated by negative pressure dialysis against Buffer b.

As a final purification step, the concentrated fractions containing both the 72 and 73 kd proteins can be applied to a Sepharose 6B column (2.5 × 90 cm) equilibrated in Buffer b (not shown). This step provides little further purification of the proteins but does provide some side fractions that contain homogeneous 72 and 73 kd proteins. In the final pooled preparation, small amounts of contaminating proteins with molecular masses of approximately 100,000, 35,000, and 32,000 daltons were detected on our SDS-polyacrylamide gels. We do not know whether these latter polypeptides are actually associated with the 72 and 73 kd proteins or merely represent minor contaminants. A yield of approximately 2–3 mg of the 72/73 kd mixture was obtained from 3–4 g of cells (wet weight).

Analysis of the Purified 72, 73, and 90 kd Stress Proteins by Two-dimensional Gel Electrophoresis—To determine if the purified 72, 73, and 90 kd proteins as well as the 100 kd protein co-purifying with 90 kd were the same as the corresponding stress proteins made in vivo, a comparison of their characteristics with those of the proteins isolated from cells in vivo was made by two-dimensional gel electrophoresis. The purified 72, 73, and 100 kd proteins were separated on a Coomassie blue-stained SDS-polyacrylamide slab gel as shown in Fig. 7.
patterns on two-dimensional gels with the pattern of \(^{35}S\) methionine-labeled polypeptides synthesized in HeLa cells exposed to AzC was undertaken. The \(^{35}S\) methionine-labeled proteins synthesized in AzC-treated HeLa cells were combined with either the purified unlabeled 72/73 kd or with the unlabeled 90/100 kd proteins and the mixtures were analyzed by two-dimensional gel electrophoresis (Fig. 9). The gels were first stained with Coomassie blue to identify the unlabeled purified proteins and 100 kd proteins (Fig. 9B) as well as the 72 and 73 kd proteins (C). Subsequently, the two-dimensional gels were fluorographed to reveal the radio-labeled in vivo-synthesized stress proteins (A). Superimposition of the fluorograph upon the Coomassie blue-stained gel demonstrated that the purified 72, 73, 90, and 100 kd proteins co-migrated with the corresponding \(^{35}S\) methionine-labeled 72, 73, 90, and 100 kd proteins made in vivo. This then indicates that the proteins purified are the stress-induced 72, 73, and 90 kd proteins and that the proteins are not significantly altered during their purification.

**Physical Properties of the Stress Proteins**—To determine the native molecular mass of the purified stress proteins, we measured both their Stokes radii by gel filtration and their sedimentation coefficients by density gradient centrifugation (Fig. 9). The proteins were first stained with Coomassie blue to identify the unlabeled purified proteins and 100 kd proteins (Fig. 9B) as well as the 72 and 73 kd proteins (C). Subsequently, the two-dimensional gels were fluorographed to reveal the radio-labeled in vivo-synthesized stress proteins (A). Superimposition of the fluorograph upon the Coomassie blue-stained gel demonstrated that the purified 72, 73, 90, and 100 kd proteins co-migrated with the corresponding \(^{35}S\) methionine-labeled 72, 73, 90, and 100 kd proteins made in vivo. This then indicates that the proteins purified are the stress-induced 72, 73, and 90 kd proteins and that the proteins are not significantly altered during their purification.

**Table 1:** Physical properties of the stress proteins

| Stress protein | Molecular mass as determined by SDS-polyacrylamide gel electrophoresis | pl of major charge isomer as determined by isoelectric focusing* | Stokes radius | Sedimentation coefficient | Native molecular mass |
|---------------|--------------------------------------------------------------------------------|---------------------------------------------------------------|----------------|--------------------------|----------------------|
| 72 kd         | 72,000                                                                         | 5.6                                                           | 42.6*          | 4.3*                     | 73,800*              |
| 73 kd         | 73,000                                                                         | 5.6, 6.3                                                      |                |                          |                      |
| 90 kd         | 90,000                                                                         | 5.2                                                           | 69             | 5.8                      | 165,000*             |
| 100 kd        | 100,000                                                                        | 5.0                                                           |                |                          |                      |

* Data presented in Ref. 8.

* Value determined for 72/73 kd mixture (see text).

* Value determined for 90/100 kd mixture (see text).
TABLE II
Amino acid composition of the HeLa 72/73, and 90 kd stress
proteins

|        | 72/73 kd | 90 kd |
|--------|----------|-------|
| Asp"   | 13.0     | 12.7  |
| Thr    | 6.4      | 5.7   |
| Ser    | 6.6      | 6.1   |
| Gluabant | 12.1  | 18.1  |
| Pro    | 4.0      | 2.5   |
| Gly    | 9.2      | 5.2   |
| Ala    | 8.0      | 5.1   |
| Val    | 6.5      | 5.1   |
| Met    | 0.7      | 2.7   |
| Ile    | 5.4      | 5.7   |
| Leu    | 10.3     | 9.3   |
| Tyr    | 1.5      | 2.9   |
| Phe    | 3.5      | 3.4   |
| His    | 2.0      | 1.7   |
| Lys    | 7.3      | 9.7   |
| Arg    | 3.6      | 4.0   |
| Cys    | ND       | ND    |
| Trp    | ND       | ND    |

" Represents both Asp and Asn.
abant Represents both Glu and Gln.
* Not determined.

The amino acid composition of the 72/73 kd mixture and
the 90 kd protein was determined next. The proteins were
further purified by SDS-polyacrylamide gel electrophoresis,
eluted from the gel, dialyzed extensively to remove salts and
other impurities, and finally oxidized by treatment with per-
formic acid. The amino acid composition of the 72/73 kd
mixture and the 90 kd protein, as determined on a Beckman
119 CL automated analyzer, is presented in Table II. A com-
parison of the composition determined for the 72 and 73 kd
proteins agrees well with the predicted amino acid composi-
tion of the analogous 70 kd protein from Drosophila mel-
agaster (as deduced from the nucleotide sequence of the
gene (40)).

In light of the observations that the 90 kd and some of the
100 kd, as well as the 72 and 73 kd, proteins co-purified during
column chromatography and co-sedimented during equilib-
rium sucrose sedimentation, the relationship of the proteins
by one-dimensional peptide mapping was examined. Each
individual protein was further purified by SDS-polyacryl-
amide gel electrophoresis, the proteins were eluted from the
gels and iodinated, and peptide maps were generated via the
method of Cleveland et al. (32) (see “Experimental Pro-
dures” for details). Analysis of the 90 and 100 kd proteins,
digested with varying amounts of Staphylococcus aureus V8
protease and examined on a 15% SDS-polyacrylamide gel,
revealed no obvious similarities (Fig. 10). A similar analysis of
the 72 and 73 kd proteins, however, demonstrated a consid-
erable amount of homology between the two proteins (Fig.
11). (Analysis of the tryptic peptides of 72 and 73 kd proteins
by high pressure liquid chromatography revealed a similar
homology between the two proteins.) These results appear
consistent with those of Hightower and White (4) who showed
that the analogous 72 and 73 kd stress-induced proteins pres-
ent in cultured rat embryo cells are related polypeptides as

![Fig. 10](image1.png)

**FIG. 10.** Comparison of the 90 and 100 kd proteins by one-
dimensional peptide mapping. The purified 90 and 100 kd proteins,
obtained after gel filtration over Sephacryl S-300 (Fig. 5), were
prepared for one-dimensional peptide mapping as described under
"Experimental Procedures." Cleavage of the iodinated 125I-90 kd
(lanes A-E) and 100 kd (lanes F-J) proteins with S. aureus V8
protease was done by the procedure outlined by Cleveland et al. (32).
All samples were analyzed on a 15% SDS-polyacrylamide gel. Shown
is the autoradiograph of the gel. Lane A, 90 kd protein, no protease.
Lane B, 90 kd protein digested with 0.5 µg of protease. Lane C, 90 kd
protein digested with 0.1 µg of protease. Lane D, 90 kd protein
digested with 0.05 µg of protease. Lane E, 90 kd protein digested
with 0.01 µg of protease. Lane F, 100 kd protein, no protease. Lane G, 100
kd protein digested with 0.5 µg of protease. Lane H, 100 kd protein
digested with 0.1 µg of protease. Lane I, 100 kd protein digested
with 0.05 µg of protease. Lane J, 100 kd protein digested with 0.01 µg of
protease.

![Fig. 11](image2.png)

**FIG. 11.** Comparison of the 72 and 73 kd proteins by one-
dimensional peptide mapping. The purified 72 and 73 kd proteins,
obtained after gel filtration over Sepharose 6B-CL, were prepared for
one-dimensional peptide mapping as described under “Experimental
Procedures.” Cleavage of the iodinated 125I-proteins with S. aureus
V8 protease was done by the procedure of Cleveland et al. (32).
Samples were analyzed on a 15% SDS-polyacrylamide gel. Shown is
an autoradiograph of the gel. Lane A, 72 kd protein, no protease.
Lane B, 72 kd protein digested with 0.1 µg of protease. Lane C, 72
kd protein digested with 0.05 µg of protease. Lane D, 72 kd protein
digested with 0.05 µg of protease. Lane E, 72 kd protein digested
with 0.01 µg of protease. Lane F, 73 kd protein digested with 0.1 µg of
protease. Lane G, 73 kd protein digested with 0.01 µg of protease. Lane H,
73 kd protein digested with 0.05 µg of protease. Lane I, 73 kd
protein digested with 0.01 µg of protease. Lane J, 73 kd protein, no protease.
determined by one-dimensional peptide mapping. It would appear then that the 90 and 100 kd proteins are not related to one another while the 72 and 73 kd proteins are similar but not identical polypeptides.

**DISCUSSION**

The phenomenon of stress (or heat shock) in eukaryotes involves a specific set of coordinate changes within the cell. While considerable work has focused on the genes induced and their corresponding mRNAs synthesized during the stress response, only recently has much attention been paid to the stress proteins themselves in terms of their structure, intracellular location, and function. To these ends then, we have begun purifying the six major HeLa stress proteins and here we report the purification of three of the six. A schematic outline of this purification is presented in Fig. 12.

Growth of suspension HeLa cells for periods of 4 to 16 h in medium containing 5 mM A2C, an amino acid analogue of proline, results in the induction of six proteins with apparent molecular masses of 72, 73, 80, 90, 100, and 110 kilodaltons as determined by their migration in one- and two-dimensional polyacrylamide gels (e.g. Ref. 9). Similar induction of these proteins is observed in cells grown in medium containing 0.25 mM ZnCl₂ or grown under heat shock conditions. The enhanced synthesis of the same six proteins also occurs in chick embryo fibroblasts, in gerbil fibroma cells, and in baby hamster kidney cells grown under stress (data not shown). The similarities in the molecular masses of the stress proteins induced in these different cell lines indicates that the response to altered growth conditions in vitro is apparently well conserved.

Fractionation by differential sedimentation of HeLa cells grown under heat shock revealed that most of the 90 kilodaltons and about one-half of the 72 and 73 kd stress proteins partition into the soluble phase following lysis of the cells in low ionic strength buffer. Conversely, it was found that the particulate fraction after centrifugation of the Dounce-homogenized cell lysate was enriched in both the 80 and 100 kd stress proteins. Some portion of both the 80 and 100 kd proteins, however, was also present in the supernatants following the low and high speed centrifugations. The presence of the 100 kd protein in the particulate fraction is consistent with our recent observation that this protein is located, as determined by immunofluorescence, in or near the Golgi apparatus in a number of different cell types so far examined. The observation that some of the 72 and 73 kd proteins fractionate with the low speed pellet is consistent with the findings of others (25, 39) that a portion of the apparently analogous 70 kd heat shock proteins of D. melanogaster cells appear to be present in the nucleus.

Because of the interest in determining the identity, intracellular location, and function of the stress proteins, it seems appropriate here to discuss what is presently known about these proteins. First, it seems clear that all of the stress-induced proteins (with a few minor exceptions) are present in tissues and in undamaged "normal" tissue culture cells (4, 11). While the 72, 80, 100, and 110 kd proteins are present in apparently low amounts, the 72, 73, and 90 kd proteins appear as prominent proteins in a variety of different cell types grown in vitro. The effect of growing cells in tissue culture, however, may itself be a stressful situation and thereby result in a slight induction of these proteins as compared to the in vivo tissue. Nevertheless, it would appear reasonable to assume that all of these proteins, in addition to serving in the stress response, perform some function in the normal cell. Secondly, analysis of stressed HeLa cells labeled with H₃²PO₄ has shown that the 80, 90, and 100 kd proteins are all phosphoproteins. It is not as yet clear what changes, if any, occur in the states of phosphorylation of these three proteins during the stress response.

With regard to the 90 kd protein, a number of laboratories have observed that a portion of the transforming protein of Rous sarcoma virus, pp60⁶⁰⁰⁰, appears to exist as part of a complex with both a 80 kd protein and the 90 kd stress protein (34-36). More recently, Adkins et al. (37) have shown that a fraction of the putative transforming proteins of PRC11 avian sarcoma virus, p105 and p110, similarly exist in a complex with a 50 kd protein and the 90 kd stress protein. In light of these reports detailing the associations of 90 kd with other cellular proteins, the observation that small amounts of the 100 kd stress protein (which is mainly associated with the Golgi apparatus) co-purify with the 90 kd protein is most intriguing. The significance of these findings concerning the existence of various complexes containing the 90 kd protein, however, is still unknown. Interestingly, it also has been reported that the 90 kd protein in crude extracts of chicken cells appears to have, upon gel filtration, an M₉ = 560,000 (11). Although we find a lower value for the native molecular mass of the purified 90 kd protein (165,000 daltons), the higher value reported may reflect an association of the 90 kd protein with other proteins in such crude extracts.

Concerning the subcellular location of the stress proteins, a number of groups have observed a migration of the newly synthesized stress proteins to the nucleus in stressed Drosophila cells (21-25). In most of these studies, proteins with a molecular mass of approximately 70,000 daltons were found

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to be enriched in the nuclear region. Additionally, Wang et al. (38) have reported that two apparent heat shock proteins of approximately 68,000 daltons are associated with the cytoskeletal network in both avian and mammalian cells. The authors also reported that these proteins are methylated in both normal and heat-shocked cells. The possible relationship of these proteins to the 72/73 kd stress proteins described here is currently being examined.

Purification of the remaining three HeLa stress proteins, 80, 100, and 110 kd, is in progress in our laboratory. We have recently made antibodies in rabbits against each of the six HeLa stress proteins and are currently examining their intracellular location by immunofluorescence. With both the purified proteins as well as their corresponding antibodies available, it may be possible to determine both the location and eventually the function of the stress proteins.

Acknowledgments—We are indebted to J. D. Watson for his enthusiastic support and encouragement of this work. Special thanks to G. P. Thomas for many helpful and stimulating discussions. We also thank J. I. Garrels for performing the two-dimensional gel analyses.

The technical assistance of B. McLaughlin, P. Renna, and M. Szadkowski is greatly appreciated.

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