# Ultrastructural and Biochemical Analysis of the Stress Granule in Chicken Embryo Fibroblasts

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*Abstract.* The ultrastructure and biochemical composition of cytoplasmic particles that form in chicken embryo fibroblasts during stress have been analyzed. We showed previously that these particles contained the small stress protein, sp 24, and antibodies specific to sp 24 were used here to identify the stress granule. In thin sections, the stress granule was a densely staining, membraneless, cytoplasmic body and appeared as a highly condensed area of cytoplasm in freeze-fracture preparations. Hypotonic swelling of cells before freeze-fracture analysis revealed a basketlike structure composed of interconnecting protein cables. No other proteins could be cross-linked to sp 24 when stress granules were treated with dithiobis-(succinimidyl propionate). High resolution autoradiographic analysis with [$^3$H]uridine failed to identify any associated RNA synthesized in the period immediately before the stress. Thus the stress granule appears to be composed predominantly of sp 24 aggregates. Sp 24 could be purified to homogeneity from the stress granule by solubilization in 8 M urea and anion exchange chromatography.

VIRTUALLY all organisms respond to an environmental stress by redirecting their protein synthetic machinery to produce a small set of proteins. These proteins are classically termed heat shock proteins but are now called stress proteins since it is well established that a wide range of stressors such as amino acid analogues, oxidants, heavy metals, and ethanol induce the same set of proteins (reviewed in references 2, 14, 34, and 39). In chicken embryo fibroblasts (CEF),[1] the subunit molecular weights of the major stress proteins are 89,000, 70,000, 24,000, and 8,000. Antibodies against the two higher molecular weight chicken stress proteins recognize similar sized stress proteins in widely diverse organisms from insects to humans (25). In addition, the amino acid sequence derived from the sequence of the gene encoding the 70,000-mol-wt stress protein (sp 70) from human cells (21) is 50% homologous with that of the *Escherichia coli* dnaK gene, which codes for a 70,000-mol-wt stress protein (31). The 8,000-mol-wt protein is ubiquitin, a very highly conserved protein found in all eukaryotic cells (5).

The current state of knowledge of the structure and function of the stress proteins has been summarized recently (38). The sp 70 is a member of a family of ATP-binding proteins and translocates from the cytoplasm to the nucleus and nucleolus upon heat shock. Its presence in the nucleolus correlates with the recovery of normal nucleolar morphology that had been altered by a temperature stress. The sp 70 has been implicated also in an activity that dissociates stress-induced macromolecular complexes. The sp 89 protein is also a member of a gene family. In certain nonstressed cells, this protein can be detected in complexes with the pp60src tyrosine kinase, steroid receptor proteins, and actin.

Proteins similar in molecular weight to the chicken 24,000-mol-wt stress protein (sp 24) are synthesized in most species after a stress. However, these small stress proteins, which range in molecular weight from 15,000 to 30,000, are not as highly conserved interphylogenically as the other major stress proteins, and antibodies raised against the chicken sp 24 do not cross-react with proteins from non-avian species (25). Comparisons of the amino acid sequences derived from the sequence of the genes encoding the small stress proteins from *Drosophila* (22), soybean (32), *C. elegans* (37), *Xenopus laevis* (3), and HeLa cells (20) show that these proteins contain a small region that is highly conserved and homologous to a sequence of about 75 amino acids in mammalian alpha-crystallin, a major protein of the lens.

We recently showed by subcellular fractionation and immunofluorescence microscopy that the chicken sp 89, sp 70, sp 24, and the vimentin-containing intermediate filament network undergo profound changes in their intracellular distributions as a result of stress (12). During the course of that study, we observed an unusual array of phase-dense granules appearing in the perinuclear region of CEF which had been heat shocked, allowed to recover, and subjected to a second heat shock. The granules remained insoluble when treated with nonionic detergents. The majority of the cellular sp 24 was localized to these heat shock–induced granules. To gain

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; sp 24, 24,000-mol-wt stress protein; sp 70, 70,000-mol-wt stress protein; sp 89, 89,000-mol-wt stress protein.

possible clues to the function of sp 24 during stress, we have investigated the ultrastructure of the heat shock–induced cytoplasmic granules and the biochemical properties of its major component. Our results are presented here and offer further information about the unusual properties of sp 24.

## Materials and Methods

### Cell Culture and Stress Conditions

Secondary CEF, prepared from 11-d chicken embryos as described previously (24), were grown almost to confluence on culture dishes in MEM supplemented with 3% FCS. For obtaining restressed CEF, the medium of cells was replaced with MEM supplemented with 3% FCS and 10 mM Hepes, pH 7.4, and cell cultures were placed in a 45°C cell incubator for 3 h. Cell survivability was assayed by trypan blue dye exclusion and, under these heat shock conditions, over 90% of CEF survived the stress. The medium was replaced with fresh MEM supplemented with 3% FCS and cultures were allowed to recover overnight at 37°C before a second incubation at 45°C for 3 h.

### Immunogold Labeling

All procedures were performed at 4°C unless otherwise noted. The cell monolayer was washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$) and either fixed by a 2-h incubation in periodate-lysine-paraformaldehyde solution (0.01 M $NaIO_4$, 0.075 M lysine, 2% paraformaldehyde, 0.0375 M sodium phosphate, pH 6.2) followed by permeabilization with 0.5% Triton X-100 in PBS for 10 min, or permeabilized first with 0.5% Triton X-100 in PBS for 10 min, then washed with PBS before periodate-lysine-paraformaldehyde fixation. The fixed cells were preincubated overnight with 0.01% gelatin in PBS containing 0.02% azide (gelatin/PBS) then incubated for 20 h with rabbit anti–CEF sp 24 antibody (0.14 mg/ml) in gelatin/PBS solution. After washing with gelatin/PBS, the monolayer was incubated overnight with goat anti-rabbit IgG conjugated with 5-nm gold (Janssen Life Sciences Products, Piscataway, NJ) and washed with gelatin/PBS before processing for electron microscopy. Cells were postfixed with 1% osmium tetroxide in 0.15 M sodium cacodylate, incubated with 4% uranyl acetate at room temperature, dehydrated with ethanol, and embedded in Polybed. Sections were stained with 4% uranyl acetate and lead citrate and viewed in a Zeiss 10A electron microscope.

### Electron Microscopy of CEF

Secondary CEF were grown on coverslips and were restressed immediately before rapid freezing. The frozen samples were fractured, deep-etched, and rotary-replicated according to Heuser (19). For analysis of nuclear/cytoskeletal fractions, CEF were lysed with 70 mM KCl, 30 mM Hepes, pH 7.0, 5 mM $MgCl_2$, 3 mM EGTA (buffer A) containing 0.5% Triton X-100 and processed as described by Heuser and Kirschner (18, 19). Analysis of purified sp 24 aggregates was performed according to Heuser (17).

### Immunoselection of Stress Granules

The nuclear/cytoskeletal fraction from restressed CEF, prepared as described above, was resuspended in 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 50 mM Tris-Cl, 150 mM NaCl, pH 7.4, and sheared by repeated pipetting. It was then centrifuged at 1,500 $g_{max}$, the pellet discarded, and the supernatant was incubated with *Staphylococcus aureus* pretreated with anti-sp 24 antibody fraction. After 30 min at room temperature, the antibody-antigen bacteria complexes were pelleted at 1,500 $g_{max}$, thoroughly washed with the same buffer, and then with 80 mM KCl. These samples were processed for freeze-fracture, deep-etch replica formation (18).

### Solubility of sp 24

Release of sp 24 from the stress granule was measured by obtaining a nuclear/cytoskeleton fraction from restressed CEF, which were prepared as described above except that a portion of the cell cultures was shifted to a medium of MEM lacking methionine, supplemented with 1% FCS, and labeled with 25 μCi of [$^{35}$S]methionine (>1,300 Ci/mmol; Amersham Corp., Arlington Heights, IL) per $10^7$ cells during the first 3 h of the recovery period after the initial stress. Nuclear/cytoskeletal fractions were prepared by lysing monolayers with 0.5% Triton X-100 in PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The detergent-insoluble nuclear/cytoskeletal fraction was washed once with PBS and treated with various reagents as described in Table I. The incubation solution was collected, and the material remaining in the dishes was washed once with the same reagent. This wash was combined with the incubation solution. The nonreleased material was solubilized in Laemmli sample buffer, and an equal proportion of both fractions were analyzed by SDS-PAGE on 11% polyacrylamide gels (28) followed by fluorography (6, 29). Bands corresponding to sp 24 were excised from the gel, added to Scinti-Verse E (Beckman Instruments, Inc., Palo Alto, CA), and counted on a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

Phase separation of sp 24 in Triton X-114 was measured by the procedure of Bordier (7) on the cytosolic fraction obtained from sonically disrupted cells that were heat shocked at 45°C for 3 h and then radiolabeled with 25 μCi [$^{35}$S]methionine per $10^7$ cells at 37°C for 3 h.

*Table I. Solubility of HSP 24*

A. Extractability of sp 24 from the stress granule*

| Treatment | Fraction solubilized |
|---|---|
| | % |
| 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 5 mM KCl, 3 mM MgCl | 8 |
| +0.5% NP-40 | 6 |
| +10 mM EDTA | 7 |
| +RNase (0.2 μg/μl, 30 min, 37°C) | 8 |
| +DNase (0.2 μg/μl, 30 min, 37°C) | 7 |
| +ATP, ADP, AMP, dATP, AMPPMP, ATP-γS, or GTP (1 mM, 30 min, RT) | <10 |
| 50 mM Tris-Cl, pH 7.4 | 5 |
| +10% Glycerol | 3 |
| +25 mM 2-Mercaptoethanol | 34 |
| +1% SDS | 88 |
| +8 M Urea | 88 |
| 5 mM Tris-Cl, pH 7.4 | 19 |
| 100 mM Acetic acid (pH 3.1) | 72 |
| 100 mM Sodium carbonate (pH 9.1) | 66 |
| 100 mM HCl (pH 1.3) | 86 |

B. Partitioning of sp 24 during Triton X-114 phase separation‡

| Treatment | Fraction in detergent phase |
|---|---|
| | % |
| 1% Triton X-114 in 150 mM NaCl, 10 mM Tris-Cl, pH 7.4 | 62 |

* The nuclear-cytoskeletal fraction from $10^6$ cells was incubated with 200 μl of the reagent overnight at 4°C unless otherwise noted. Samples were prepared as described in Materials and Methods.

‡ Heat-shocked CEF were sonicated and the soluble fraction subjected to phase separation with 1% Triton X-114 according to Bordier (7). Proteins in the aqueous and detergent phases were acetone precipitated and equal amounts of samples from both phases were analyzed by SDS-PAGE and fluorography.

### Purification of sp 24

Sp 24 was obtained from restressed CEF grown in 850-$cm^2$ culture bottles. The cultures were heat shocked, allowed to recover, and restressed according to the schedule described above. The cells were washed three times with ice cold PBS, harvested, and lysed with 0.5% Triton X-100 in PBS containing 0.1 mM PMSF (1 ml per $10^7$ cells). The insoluble nuclear/cytoskeletal fraction was collected by centrifugation at 1,500 $g_{max}$ for 5 min and washed twice with PBS and once with 25 mM Tris-HCl, pH 7.2. This fraction was treated with 8 M urea, 25 mM Tris-HCl, pH 7.2 (buffer B) at 4°C for 5–15 h. The solubilized material was applied to a DE52 column (Whatman Inc., Clifton, NJ) equilibrated in buffer B (~1 ml bed vol per $10^8$ cells). The column was washed with 10 bed vol of buffer B and eluted with a linear gradient of 0–0.2 M KCl in buffer B at a flow rate of ~10–20 ml/h. Fractions were monitored for radioactivity, and protein content was analyzed by SDS-

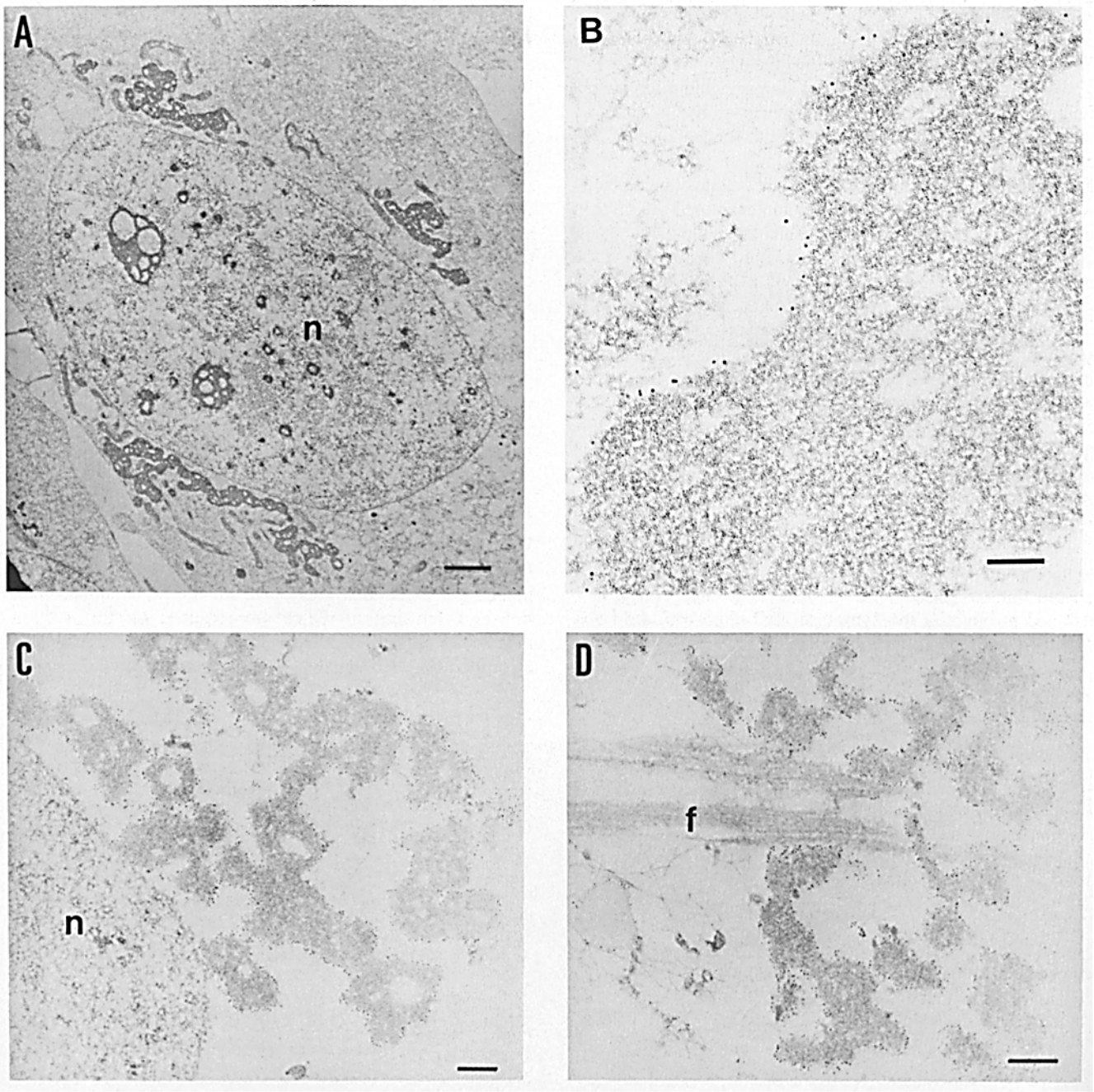


*Figure 1.* Ultrastructural localization of sp 24 in restressed CEF and in nuclear/cytoskeletal fractions. Restressed CEF were fixed with periodate-lysine-paraformaldehyde solution before (*A* and *B*) or after (*C* and *D*) treatment with nonionic detergent, followed by incubation with anti-sp 24 antibody and gold-conjugated anti-rabbit antibody. To enhance diffusibility of the second antibody, a gold particle size of 5 nm was used. A portion of a stress granule is enlarged in *B* to show gold particles bound to its periphery. Nuclei are labeled *n* and filament bundles, *f*. Few gold particles were observed in restressed CEF not treated with anti-sp 24 antibody. Bars: (*A*) 2 μm; (*B*) 0.1 μm; (*C* and *D*) 0.25 μm.

PAGE on 11% polyacrylamide gels according to Laemmli (29). Fractions containing only sp 24 were pooled.

### Amino Acid Analysis

The amino acid composition of sp 24 was derived from 24, 48, and 72 h hydrolyses in 6 N HCl in vacuo at 110°C after performic acid oxidation for determination of cysteine and methionine. Analyses were performed by the Protein Chemistry Facility, Washington University School of Medicine, St. Louis, MO, on a Waters Associates (Milford, MA) HPLC with model 840 control and data reduction. Amino acids were detected and quantified by post column derivitization using *o*-phtalaldehyde and continuous infusion of hypochlorite for proline detection. Threonine and serine values were calculated after extrapolation to zero time hydrolysis. Tryptophan was not determined.

## Results

### Ultrastructural Localization of sp 24

Our previous immunofluorescence studies showed that CEF sp 24 localized to perinuclear phase-dense structures when

cells were restressed by either a heat shock or arsenite treatment (12). We term these irregular structures stress granules. To characterize the stress granule at the ultrastructural level, we used anti-sp 24 antibodies and immunogold microscopy. Restressed CEF were treated with periodate-lysine-paraformaldehyde fixative, permeabilized with 0.5% Triton X-100, and incubated with anti-sp 24 antibody followed by 5-nm gold-labeled anti-rabbit antibody. The gold-labeled cells were processed for thin sectioning and examined by electron microscopy. Dense and irregularly shaped cytoplasmic particles were observed in a low magnification micrograph of the restressed CEF (Fig. 1 *A*). As shown in Fig. 1 *B*, the anti-sp 24 antibody was concentrated about the periphery of these cytoplasmic particles. These particles were identified as the stress granules by the following criteria: (*a*) they were the only structures labeled with anti-sp 24 antibody; (*b*) they were localized in a perinuclear array; and (*c*) they were not observed in unstressed cells. These micrographs show further that the stress granule has a structure similar to that of the heat-stressed nucleolus in that both are membraneless, dense structures with less dense "pockets." No gold was observed inside the stress granule even though the particle is not bound by a lipid bilayer. Either sp 24 is localized only to the surface of the stress granule or the antibodies are excluded due to steric limitations. Little gold label was distributed throughout the cytoplasm and almost no gold was found in samples not incubated with anti-sp 24 antibody.

We also examined the ultrastructural localization of sp 24 in restressed cells that had been permeabilized with Triton X-100 (0.5%) before periodate-lysine-paraformaldehyde fixation. The immunogold-labeled structures again appeared very dense (Fig. 1, *C* and *D*) but not identical to those seen in fixed whole cells (Fig. 1 *B*). First, they appeared spread apart as if the lysis had unfolded or dissociated part of the structures noted in whole cells. Second, the stress granules were more heavily labeled with gold which, presumably, is due to enhanced diffusability of the antibodies. However we cannot rule out the possibility that the stress granule has undergone detergent-mediated conformational changes, which exposed more antigenic sites. Many of these gold-labeled structures contained clear cavities but an occasional cavity was immunolabeled with gold, suggesting that these areas were opened by the detergent treatment instead of being penetrated by antibody. The nucleus (Fig. 1 *C*) and bundles of filaments (Fig. 1 *D*) were retained in the preparation but were unlabeled by gold when tested with antibodies specific for sp 24.

### *Freeze-fracture Analysis of Restressed CEF*

We sought additional ultrastructural information by using a freeze-fracture, deep-etch technique. This procedure avoids potential artifacts that may occur during the chemical fixation and dehydration which are required for thin-section preparations. Restressed cells treated by the freeze-fracture technique revealed unusual areas of condensed or collapsed

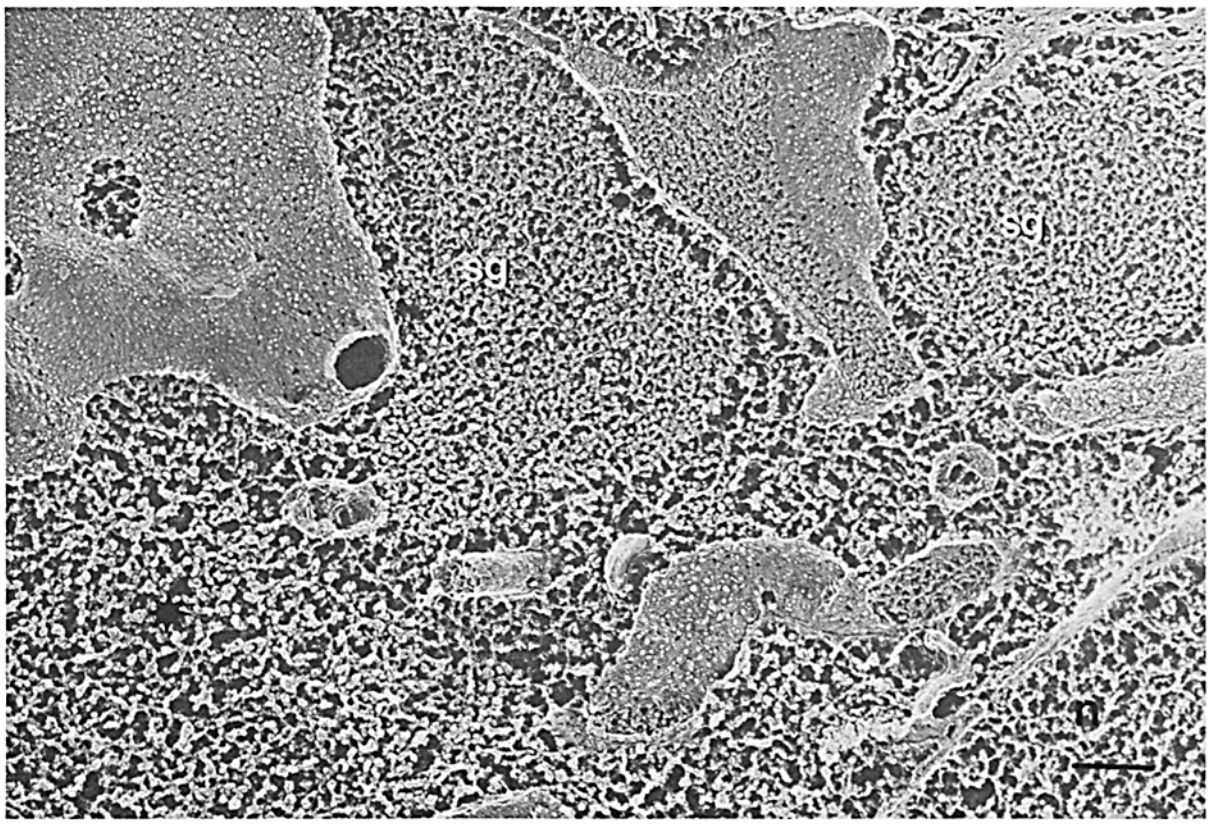


*Figure 2.* Ultrastructure of restressed CEF. The ultrastructure of restressed CEF was examined by freeze-fracture, deep-etch replica formation technique. Abnormally concentrated areas of cytoplasm are labeled *sg* (stress granule), an area typical of normal cytoplasm, *, and the nucleus, *n*. Bar, 0.2 μm.

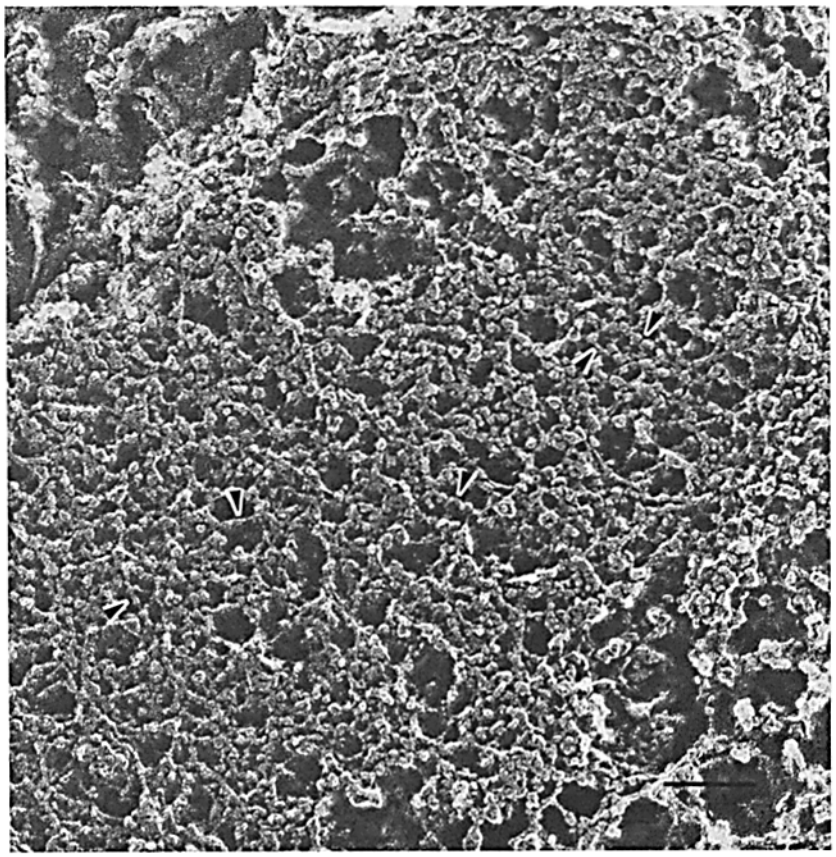

*Figure 3.* Ultrastructural analysis of the stress granule from hypotonically swollen restressed CEF. Restressed CEF were briefly treated with PBS diluted fivefold with water before freeze-fracture, deep-etch platinum replication. Arrowheads highlight representative fibers contributing to ordered substructure. Bar, 0.1 μm.

cytoplasm containing dense concentrations of protein that varied in size and shape without a highly ordered internal morphology (Fig. 2). Many had a globular shape while others appeared tortuous, possibly representing clusters of globular areas. Some exhibited variation in the density of protein contained within these structures and some structures appeared to contain central cavities, again reminiscent of a nucleolar morphology. No structures of this kind were observed with control cells, and we tentatively identified these as stress granules. We could see more structure by

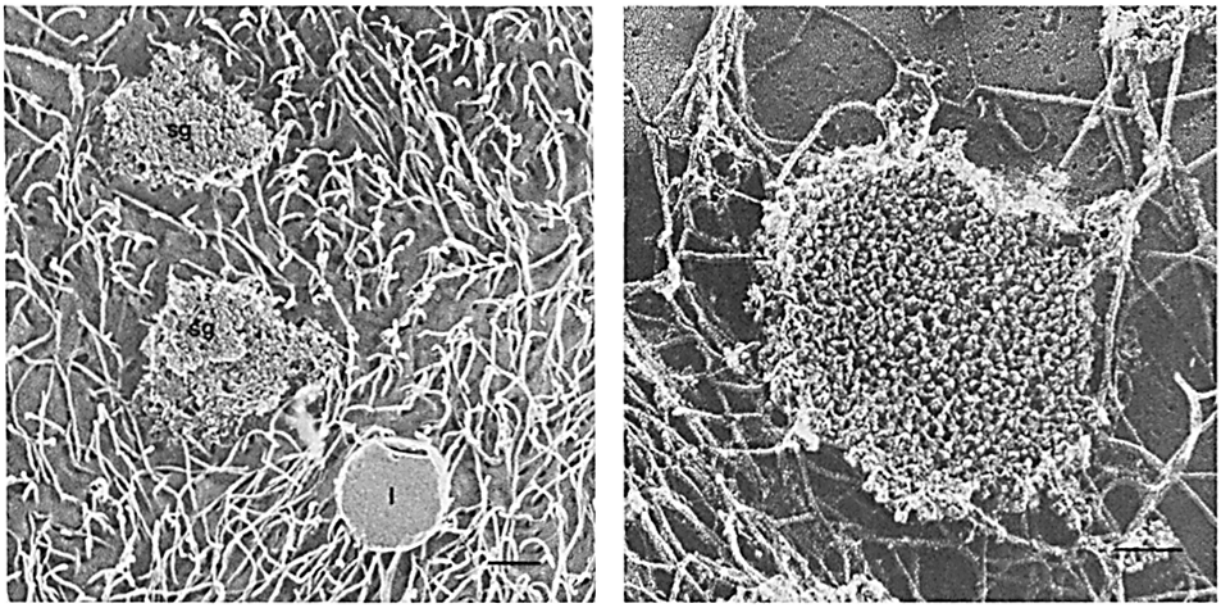


*Figure 4.* Freeze-fracture analysis of nucleus/cytoskeleton from restressed CEF. Two views of fractured stress granules (*sg*) embedded in the cytoskeleton are shown. A lipid droplet that remained associated with the cytoskeleton after fractionation is labeled *l*. Bars: (*A*) 0.2 μm; (*B*) 0.1 μm.

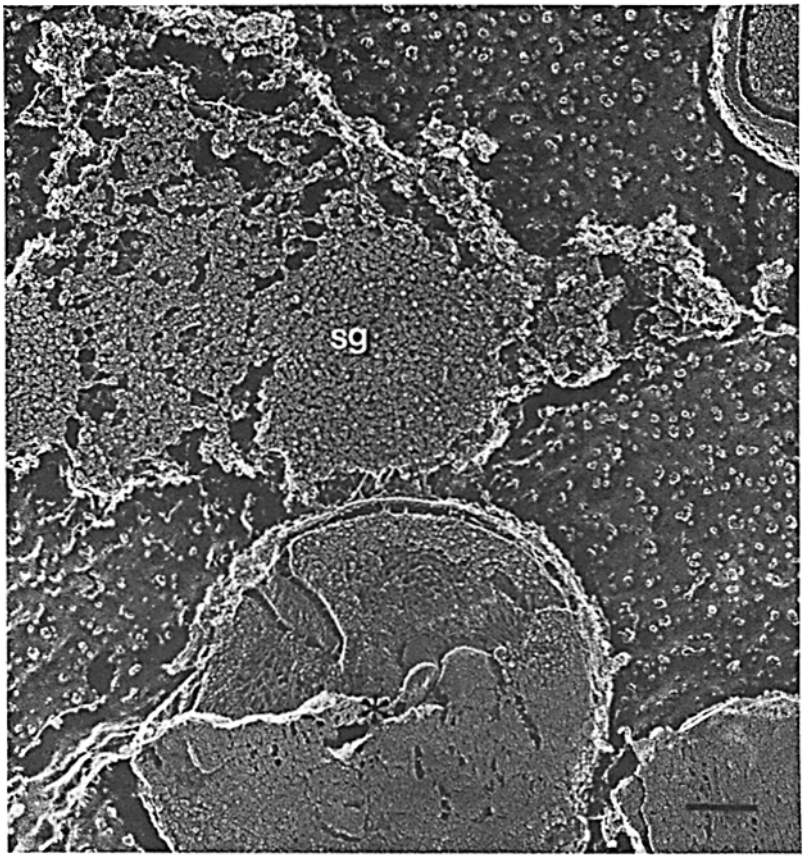


*Figure 5.* Immunoselection of stress granules. Nuclear/cytoskeletal fractions were incubated with anti-sp 24 antibodies and *Staphylococcus aureus*. The resultant immune precipitate was subjected to freeze-fracture, deep-etch replica formation analysis. The bacteria is labeled *, and the stress granule, *sg*. Only bacteria was observed in control samples not incubated with anti-sp 24 antibody. Bar, 0.2 μm.

hypotonically treating the restressed CEF before freeze-fracture and replica preparation. Instead of the dense concentration of protein, the hypotonically swollen structure appeared to be organized into loose scaffolds with parallel strands of beads with thinner cross-connections (Fig. 3).

Even greater detail of the stress granules' structure could be obtained by taking advantage of the fact that stress granules remained insoluble when restressed CEF were treated with nonionic detergent to prepare nuclear/cytoskeletons. Restressed cells, grown on glass coverslips, were treated with 0.5% NP-40 in buffer A, fixed with 2% glutaraldehyde, freeze-fractured, deep-etched, and rotary-shadowed for platinum replica formation (18, 19). In these preparations, the prelysis treatment removed most of the cytoplasmic components thus highlighting the insoluble aggregates of protein. The putative stress granules now appeared as sheets of interconnected proteins surrounded by filaments (Fig. 4). Definitive evidence that the structures observed in these preparations were in fact the stress granule was obtained by using an immunoselection procedure employing antibodies specific to CEF sp 24 which were bound to formalin-fixed *Staphylococcus aureus* (refer to Materials and Methods). Particles morphologically similar to those seen in replicas of fractured whole cells and in fractures of the nuclear/cytoskeletal fraction were bound to the bacteria (Fig. 5). In control samples where the bacteria was not pretreated with anti-sp 24 antibody, no particles were observed.

### *Composition of the Stress Granule*

The data presented above and our earlier observations at the level of the light microscope provide convincing evidence that the stress granule is heavily enriched in sp 24 molecules. We wished to know what other components might be present along with sp 24 in the granule, and we carried out several experiments designed to test for the presence of the major cytoskeletal proteins and for recently synthesized RNA in the granule. We also looked for proteins that might be strongly associated with sp 24 by using a cleavable cross-linking reagent. In most of these studies, we labeled the cells with [$^{35}$S]methionine for a 3-h period immediately after recovery from an initial 3-h heat shock. Based on a one-dimensional SDS-PAGE analysis, this labeling protocol gives a pattern of proteins much like that synthesized in nonstressed cells except for the presence of the major heat shock proteins, which account for 10–15% of the total labeled proteins. Actin, vimentin, and tubulin are the predominant species in the population of labeled proteins.

In one set of experiments, we compared the solubilities of sp 24, actin, and vimentin in the nuclear/cytoskeletal preparations that contained most of the sp 24 in the heat shock granule. We reasoned that, if the aggregation and insolubility of sp 24 in the granule were dependent on cytoskeletal proteins and their organization, any reagent that solubilized the latter should also render sp 24 soluble. We found, however, that a high salt extraction (0.6 M KI in buffer A) solubilized 70–80% of the actin and vimentin in the preparation but only 20% of sp 24. Almost all of the sp 24 and vimentin could be solubilized in the presence of 0.1% sarkosyl, a reagent used to extract intermediate filaments (8), but only 40% of the actin was released with this reagent. These results indicate that the structure of sp 24 in the granule is independent of the microfilament and intermediate filament systems.

In addition neither of these filament components was

found associated with sp 24 in our cross-linking studies. For these analyses, we added the cleavable cross-linking reagent, dithiobis-(succinimidyl propionate) to preparations of radiolabeled nuclear/cytoskeletal fractions and then used antibodies to sp 24 to immunoprecipitate any protein that might have been linked to sp 24 (11). Examination of the immunoprecipitates by SDS-PAGE after reduction of the disulfide linker showed only sp 24 to be present. To look for proteins that were made in low amounts or turned over slowly, a similar experiment was carried out with cells that had been labeled for 19 h before the initial stress. Again, only sp 24 was detected in the SDS-PAGE analysis of the immunoprecipitates.

To determine if the stress granule was a ribonucleoprotein particle containing a newly synthesized species of RNA, we radiolabeled CEF immediately before the restress event (note that, in our system, it is the restress that leads to the rapid accumulation of stress granules) with [$^3$H]uridine after the conditions initially described by Neumann et al. for the detection of RNA in plant heat shock granules (33). CEF were plated at reduced confluence on coverslips, heat shocked 3 h at 45°C, allowed to recover 16 h at 37°C, and labeled with 10 μCi/ml [5,6]-[$^3$H]uridine (42 Ci/mmol) for 2 h at 37°C. After a 20-min chase period in the absence of labeled uridine, cells were restressed for 3 h at 45°C. Coverslips were immunolabeled with anti-sp 24 antibodies followed by rhodamine-labeled second antibodies as previously described (12) and dipped in NTB-2 (Kodak) emulsion diluted 1:1 with water. exposed for 10 d, and developed with D-19 (Kodak) developer. Nuclei were heavily labeled with black grains indicative of transcriptional activity. However, stress granules, detected by immunofluorescence, were devoid of black grains (data not presented). These results show that, unlike the plant heat shock granules, the chicken stress granule does not contain significant amounts of RNA that had been synthesized immediately before the second stress.

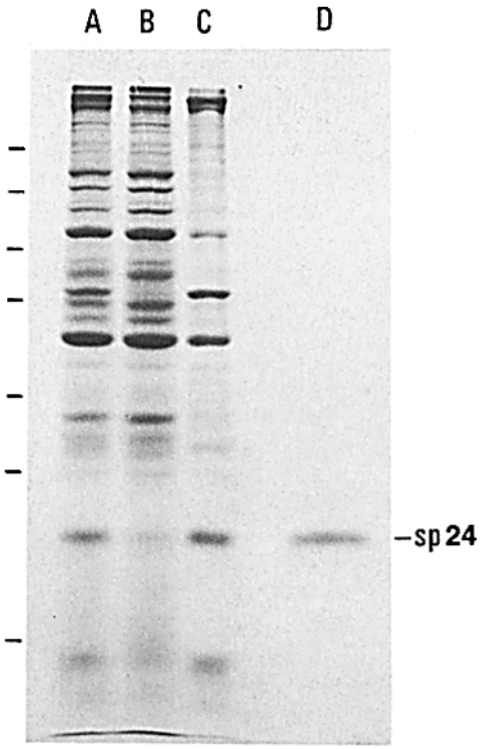


*Figure 6.* Purification of sp 24. Sp 24 was purified by chromatography on DE52 in 8 M urea from the nuclear/cytoskeletal fraction of restressed CEF. Restressed CEF, lane *A*, Triton X-100 soluble fraction, lane *B*, Triton X-100 insoluble nuclear/cytoskeletal fraction, lane *C*, and purified sp 24, lane *D*, were analyzed by SDS-PAGE and Coomassie Blue stain. Molecular weight markers are denoted by bars and correspond to α-macroglobulin (180,000), β-galactosidase (116,000), fructose-6-phosphate kinase (84,000), pyruvate kinase (58,000), fumarase (48,500), lactic dehydrogenase (36,500), triosephosphate isomerase (26,600), and β-lactoglobulin (18,400).

### *Biochemical Analysis of the sp 24*

All of the data presented thus far suggest that the stress granule is predominantly if not exclusively an aggregate of sp 24. When initially synthesized in heat stressed CEF, however, sp 24 is recovered in the soluble cytosolic fraction of cell extracts, even though in these fractions the sp 24 is in higher molecular weight species (12, 23). We wished to measure properties of the sp 24 in both its soluble, cytoplasmic form as well as in the insoluble granule, and have carried out a number of experiments to determine what reagents can release sp 24 from the granule and what properties of the soluble sp 24 could account for its propensity to aggregate. The first set of experiments is summarized in Table I. As expected, strong denaturants such as 1% SDS, 0.1% sarkosyl, low pH, and 8 M urea solubilized most sp 24 from stress granules. Incubation with 2-mercaptoethanol, higher pH (9.1), or low salt solubilized a portion of sp 24 but was less effective than strong denaturants. Most sp 24 was retained in the nuclear/cytoskeletal fraction when incubated with nucleotides, divalent cations, and nucleases (both RNase and DNase).

In vivo, the stress granule dissociates when stressed cells are allowed to recover and sp 24 is extracted as a "soluble" cytosolic protein (our previous data, 12). This soluble form of sp 24 was tested for its hydrophobicity using the method of phase separation in Triton X-114 solutions and, interestingly, was found to partition into the detergent phase (Table I B). We conclude that sp 24 is an amphiphilic protein, and this property could contribute to its aggregation into the stress granule. The hydrophobicity is unlikely to be due to covalently bound lipid since we were unable to label sp 24 with [$^3$H]palmitate or myristate during its synthesis in the stressed cell (data not shown).

Based on the results that indicated that sp 24 was the major component of the stress granule, we explored a new approach for purifying sp 24. In fact, a homogeneous preparation of sp 24 could be obtained from the detergent-insoluble nuclear/cytoskeletal fraction of restressed CEF using anion exchange chromatography in the presence of 8 M urea. The granule was treated with 8 M urea and the urea-solubilized protein was directly loaded onto a DE52 column under conditions in which sp 24 bound to the column. Upon elution with a KCl gradient, sp 24 eluted at low salt concentrations (~0.03–0.05 M KCl), followed by sp 70 (~0.04–0.07 M KCl), vimentin (~0.08–0.11 M KCl), and actin (~0.1–0.15 M KCl). Fractions of sp 24 exclusive of other proteins as assayed by SDS-PAGE were pooled (Fig. 6, lane *d*). Approximately 200 μg of highly purified sp 24 were obtained from $4 \times 10^8$ cells.

We used several methods to remove the urea from the purified protein in an attempt to obtain a soluble form of sp 24, which might then be studied with respect to forming higher molecular weight aggregates of ordered structure.

*Table II. Amino Acid Composition of sp 24*

| Amino acid | No. of residues* |
|---|---|
| Aspartic acid | 17 |
| Threonine | 7 |
| Serine | 24 |
| Glutamic acid | 37 |
| Proline | 14 |
| Glycine | 16 |
| Alanine | 26 |
| Cysteine | 2 |
| Valine | 23 |
| Methionine | 8 |
| Isoleucine | 4 |
| Leucine | 17 |
| Tyrosine | 2 |
| Phenylalanine | 7 |
| Lysine | 12 |
| Histidine | 3 |
| Arginine | 11 |

* Based on a molecular weight of 24,000.

When the denaturant was slowly removed by dialysis against successively lower concentrations of urea over a period of 24 h, a very dense, totally amorphous structure formed. Rapid removal of urea by dilution with nondenaturing buffers led to aggregation of sp 24. By electron microscopic analysis, these aggregates appeared to be proteinaceous particles that ranged in size from ~5–10 nm to >100 nm but did not resemble the structure of the stress granule. The small 5–10-nm globular particles were probably sp 24 oligomers of four to eight subunits and the rest represented various higher aggregation states of the protein. When a purified preparation of denatured sp 24 was adsorbed to mica in the presence of 8 M urea and washed to remove urea, globular particles ~10 nm in diameter were revealed by quick-freeze, deep-etch analysis to be decorating the mica flakes.

An amino acid composition of sp 24 purified from the stress granule was determined (Table II). We have compared this composition to the predicted amino acid compositions derived from gene sequence analyses of small molecular weight stress proteins from *Drosophila* (22), *C. elegans* (37), HeLa cells (20), and soybean (33). The content of proline, charged amino acids, and low level of cysteine is similar in all compositions. The methionine content is similar to that of *Drosophila*, *C. elegans*, and soybean, and higher than the characteristically low content found in mammalian cells. Attempts to sequence sp 24 were unsuccessful, indicating that the $NH_2$ terminus was blocked. The block probably did not result from cyanylation of sp 24 purified in the presence of urea since attempts to sequence sp 24 purified from nuclear/cytoskeletal fraction by preparative electrophoresis also were unsuccessful. A major high molecular weight polypeptide (20,000) obtained after cyanogen bromide cleavage of sp 24 (13) also had a blocked amino terminus.

## Discussion

Our attempts to define properties of the chicken cell sp 24 have led to several interesting new observations about this protein. Analyses at the ultrastructural level have shown that this protein can appear in membraneless, structured aggregates (termed stress granules) in the cell cytoplasm that resemble structures observed for the nucleolus in the stressed cell. These granules appear not to be linked to any of the cytoskeletal elements in the cell, and attempts to find other cellular proteins or newly synthesized RNA in them were unsuccessful. Based on these studies, we tentatively conclude that stress granules are almost exclusively arrays of the sp 24 protein. In the cell, these arrays are dissociable and the sp 24 can be isolated in a soluble form. The soluble sp 24 has amphiphilic properties, based on its distribution in a binary lipid–aqueous phase system. Sp 24 also can be solubilized from the stress granule, but this requires using a strong protein denaturant and removal of the latter leads to reaggregation of the protein. Nevertheless, we have used the latter solubilization method to obtain homogeneous preparations of the protein and this allowed for amino acid analysis. The purified protein could not be sequenced presumably because its amino terminus is blocked. Previous attempts to sequence sp 24 that had been purified by SDS-PAGE also indicated a blocked amino terminus.

At early times after a stress almost all of sp 24 can be isolated from the cell in a "soluble" cytoplasmic fraction. Even in these fractions, however, the protein is present in higher molecular weight forms—a substantial amount has an apparent molecular weight of 180,000 based on size-exclusion chromatography (23). Our earlier attempts to concentrate and purify sp 24 from these cytosolic fractions led to insoluble aggregates of the protein. We believe that the intracellular aggregation is also the result of increased concentrations of sp 24, which continues to be made during the recovery of the cells from the initial stress. The second stress appears to accelerate aggregation, perhaps as a result of other events that occur very shortly after a stress to chicken cells. One of these, for example, is a collapse of the intermediate filament network (12). However, we failed to find evidence for a strong association of vimentin with sp 24.

Formation of cytoplasmic particles of the type described here have been detected in heat shocked cells from widely divergent species. An early report on the effects of supraoptimal temperature in HEp-2 cells revealed that cytoplasmic dense material formed after subjecting the cultures to elevated temperatures for short periods of time (9). In heat shocked plant cells (*Lycopersicon peruvianum*), cytoplasmic particles (30–40 nm and 70–90 nm) in the form of large aggregates appear during the stress (33, 35). In *Drosophila* tissue culture cells, granules form in the perinuclear region upon heat shock (41). Like the CEF sp 24 the small stress proteins from other species are found in stress granules (33, 41), but they have also been detected in subcellular fractions that contain the nucleus (1, 26), chromatin (1), heteronuclear RNA (27), nucleoskeleton (40), and cytoskeleton (30). All of the latter could be a result of aggregates of small stress proteins cofractionating with these organelles.

A function for the small stress proteins has not yet been found. Because these proteins appear in high molecular weight aggregates and contain a stretch of amino acid sequences that is highly homologous to mammalian lens alpha-crystallin, many investigators have assumed that the small stress proteins form some kind of structure that protects cells from stress-imposed damage. Actually relatively little is known about the structure and function of the mammalian alpha-crystallins (4). Furthermore, Wistow and Piatigorsky

(42) have recently observed that several of the lens crystallins have sequences that are homologous to a variety of enzymes. In fact, there are a number of enzymes that polymerize to form molecules of very high molecular weight, and Frieden has pointed out that this latter property can provide for allosteric regulation of enzymatic activity (16). Perhaps the family of small molecular weight stress proteins are enzymes whose activity is regulated by polymerization. The enzymatic activity might be important for a cell's recovery from stress. During stress, the high molecular weight form would be more stable and recovery would lead to dissociation and activation of the enzyme. In this regard it is of interest that another major stress protein, the 70,000-mol-wt molecule, has an activity that, in the presence of ATP, dissociates protein complexes (10, 15, 36). An involvement of sp 70 in the function of the small stress proteins would make the stress response a highly interactive system used by cells to recover from effects of the stress.

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