

# Microinjection of Ubiquitin: Changes in Protein Degradation in HeLa Cells Subjected to Heat-Shock

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**Abstract.** Ubiquitin was radiolabeled by reaction with  $^{125}\text{I}$ -Bolton-Hunter reagent and introduced into HeLa cells using erythrocyte-mediated microinjection. The injected cells were then incubated at 45°C for 5 min (reversible heat-shock) or for 30 min (lethal heat-shock). After either treatment, there were dramatic changes in the levels of ubiquitin conjugates. Under normal culture conditions, ~10% of the injected ubiquitin is linked to histones, 40% is found in conjugates with molecular weights greater than 25,000, and the rest is unconjugated. After heat-shock, the free ubiquitin pool and the level of histone-ubiquitin conjugates decreased rapidly, and high molecular weight conjugates predominated. Formation of large conjugates did not require protein synthesis; when analyzed by two-dimensional electrophoresis, the major conjugates did not co-migrate with heat-shock pro-

teins before or after thermal stress.

Concomitant with the loss of free ubiquitin, the degradation of endogenous proteins, injected hemoglobin, BSA, and ubiquitin was reduced in heat-shocked HeLa cells. After reversible heat-shock, the decrease in proteolysis was small, and both the rate of proteolysis and the size of the free ubiquitin pool returned to control levels upon incubation at 37°C. In contrast, neither proteolysis nor free ubiquitin pools returned to control levels after lethal heat-shock. However, lethally heat-shocked cells degraded denatured hemoglobin more rapidly than native hemoglobin and ubiquitin-globin conjugates formed within them. Therefore, stabilization of proteins after heat-shock cannot be due to the loss of ubiquitin conjugation or inability to degrade proteins that form conjugates with ubiquitin.

**A**FTER heat treatment or exposure to various agents such as heavy metals, ethanol (2), or amino acid analogs (23), cells respond by increasing the synthesis of a small set of proteins called heat-shock proteins (HSPs)<sup>1</sup> (for review see reference 13). This response is universal from bacteria to man (28, 39) and may confer upon the organism the ability to withstand a second exposure to heat (thermotolerance). Despite increasing interest in the heat-shock response, the functions of many HSPs are poorly understood. Recently, ubiquitin was identified as an HSP in chicken embryo fibroblasts (6). Although ubiquitin is essential for ATP-dependent proteolysis in rabbit reticulocyte lysates (12, 21) and is a structural component of chromatin (7, 8), the relationship of these functions to the heat-shock response is not entirely clear. In the preceding paper (10), we studied the metabolism of injected ubiquitin in HeLa cells grown under normal culture conditions. Here, we describe changes that occur in the intracellular distribution, extent of incorporation into conjugates, and stability of injected ubiquitin in HeLa cells recovering from heat-shock. We also report on

the rate of degradation of injected and endogenous proteins in heat-shocked cells.

## Materials and Methods

### Injection into Cultured Cells

Radioiodination of ubiquitin by the Bolton and Hunter procedure (5), cell culture, and red blood cell (RBC)-mediated microinjection were performed as described in the preceding paper (10).

### Heat-Shock

8 or more hours after being injected with ubiquitin, HeLa cells were heat-shocked as follows. Cells plated at <50% confluency ( $1 \times 10^6$  cells per 25-cm<sup>2</sup> culture flask) in 2.5 ml of Ham's F-12 medium were removed from 37°C incubators and immediately immersed in a 45°C water bath. The duration of heat-shock is considered to be the time between immersion in the water bath and return to 37°C.

### Cell Viability after Heat-Shock

Viability was monitored by counting both surviving cells under phase-contrast and cell colonies present 1 wk after heat-shock. Cells shocked for 5 min resembled control cells in appearance, remained viable, and continued to grow. Cells that received a 30-min shock rounded up within 2 h, stopped growing, and eventually lysed; mortality was >50% by 48 h after

1. *Abbreviations used in this paper:* HMW, high molecular weight; HSP, heat-shock protein; RBC, red blood cell.

treatment. However, as late as 72 h after heat-shock, the remaining cells excluded trypan blue. We define heat treatments of 5 min or less to be reversible, and those of 30-min duration to be lethal. For more detailed descriptions of cell viability after heat-shock, see reference 18.

### Electrophoresis

Sample preparation and SDS PAGE analyses were performed as described in the previous paper (10). Two-dimensional PAGE was performed by the method of O'Farrell (34). Injected or pulse-labeled HeLa cells were removed from the monolayer with 0.1% trypsin in  $\text{Ca}^{++}/\text{Mg}^{++}$ -free saline, rinsed twice in PBS, dissolved in O'Farrell lysis buffer, and immediately frozen at  $-80^{\circ}\text{C}$ . After thawing, a 50- $\mu\text{l}$  sample ( $\sim 1 \times 10^6$  cells) was focused for 7,200 volt-hours; the final pH gradient ranged from 4.6 to 7.3. First-dimension gels were expelled from the glass tubes, equilibrated in SDS sample buffer, and frozen at  $-80^{\circ}\text{C}$  before analysis in the second dimension on 8.7% acrylamide gels. Molecular weights were calibrated as described (10).

### Labeling of Endogenous Proteins with [ $^{35}\text{S}$ ]Methionine

Fused or unfused HeLa cells were incubated for 20 min with methionine-free F-12 medium supplemented with 50  $\mu\text{Ci}/\text{ml}$  [ $^{35}\text{S}$ ]methionine to label proteins for two-dimensional electrophoretic analysis. Cells that had been exposed to  $45^{\circ}\text{C}$  for 5 min (5-min heat-shock) were incubated at  $37^{\circ}\text{C}$  for 2 h before labeling. For degradation experiments, HeLa cell proteins were labeled before heat-shock by incubating cells for 18 h in F-12 medium supplemented with 1  $\mu\text{Ci}/\text{ml}$  [ $^{35}\text{S}$ ]methionine (long pulse), or by incubating cells for 20 min in methionine-free F-12 medium supplemented to 50  $\mu\text{Ci}/\text{ml}$  of [ $^{35}\text{S}$ ]methionine (short pulse). After the pulse, cells were either heat-shocked as described or grown continuously at  $37^{\circ}\text{C}$ , then chased at  $37^{\circ}\text{C}$  in F-12 medium containing 20-fold the usual level of methionine. [ $^{35}\text{S}$ ]Methionine was obtained from Amersham Corp. (Arlington Heights, IL).

### Autoradiography

Autoradiography of thin sections was performed as described previously (30). The intracellular location of injected ubiquitin was determined by quantitation of the grain distribution over the nucleus and cytoplasm (see Table I). However, varying amounts of  $^{125}\text{I}$  were lost from the cells during fixation (40% loss from control cells, 20% from 5-min heat-shock cells, and 5% from 30-min heat-shocked cells). Free ubiquitin may be preferentially extracted since the amounts of radioiodine lost during fixation were proportional to the levels of free ubiquitin in the cell (see Fig. 1). Additionally, >90% of the radiolabel extracted from cells during fixation chromatographed on Sephadex G-100 with an apparent molecular weight of less than 20,000 (data not shown). Therefore, it is likely that autoradiography detects mainly ubiquitin conjugates.

## Results

### Ubiquitin Pools after Heat-Shock

In the previous paper (10), we arbitrarily assigned intracellular ubiquitin to one of three metabolic compartments: the free pool, histone conjugates, and high molecular weight (HMW) conjugates. As shown in Fig. 1 A, the partitioning of ubiquitin among these metabolic compartments changed dramatically in cells recovering from heat-shock. The proportion of ubiquitin in the free pool and in histone conjugates decreased, and nearly 90% of the labeled protein was converted to HMW conjugates. Similar results were obtained when ubiquitin was injected into HeLa cells that had been heat-shocked before fusion.

Since the synthesis of HSPs is induced after heat-shock (see reference 13), redistribution of ubiquitin might depend upon synthesis of one or more of the HSPs. To test this possibility, HeLa cells that had received an injection of ubiquitin were heat-shocked, and protein synthesis was immediately blocked with 100  $\mu\text{M}$  cycloheximide. Although incorporation of [ $^{35}\text{S}$ ]methionine was reduced by >98%, the redistri-

Table I. Ubiquitin Distribution in Heat-Shocked and Control HeLa Cells\*

	Autoradiography of sectioned cells		
	Ratio of the grain density for each cell compartment compared to the grain density for the whole cell		Number of cells counted
	Nucleus	Cytoplasm	
2 h post-injection			
Control	1.12	0.89	108
5-min heat-shock	1.30	0.86	83
30-min heat-shock	1.33	0.78	25
24 h post-injection			
Control	1.01	0.89	25
5-min heat-shock	1.17	0.92	109
30-min heat-shock	1.22	0.85	25
	Triton X-100 extraction of injected cells		
	Percent of ubiquitin in the Triton X-100-soluble fraction		
4 h post-injection			
Control		79.2	
5-min heat-shock		51.4	
30-min heat-shock		28.5	
24 h post-injection			
Control		80.2	
5-min heat-shock		80.0	
30-min heat-shock		30.7	

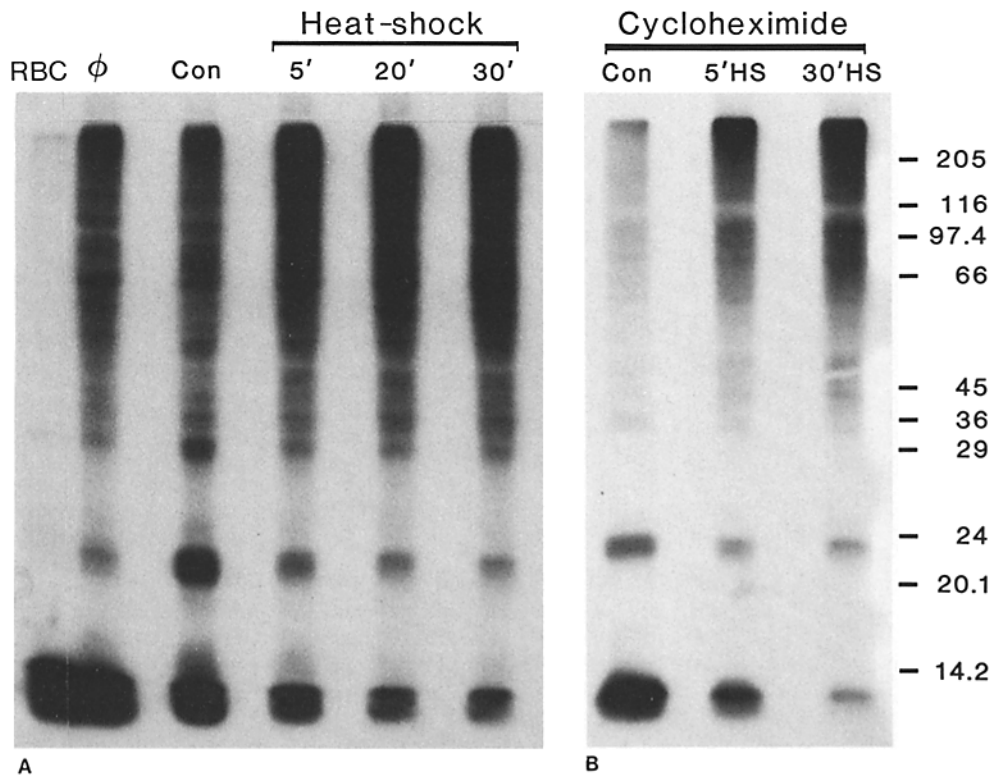
\* The intracellular location of injected ubiquitin was determined by autoradiography of sectioned cells or by extraction of cells with buffer containing 0.5% Triton X-100. Ubiquitin distribution as measured by autoradiography is the average grain density for each cell compartment divided by the average grain density over the whole cell. The distribution measured by Triton extraction is shown as the percent of radiolabel that partitions to the Triton-soluble fraction. Each value is the average from two experiments.

bution of ubiquitin to HMW conjugates was unaffected (Fig. 1 B). Hence, the increase in HMW conjugates does not require the synthesis of new HSPs.

### Relationship between Ubiquitin Conjugates and Heat-Shock Proteins

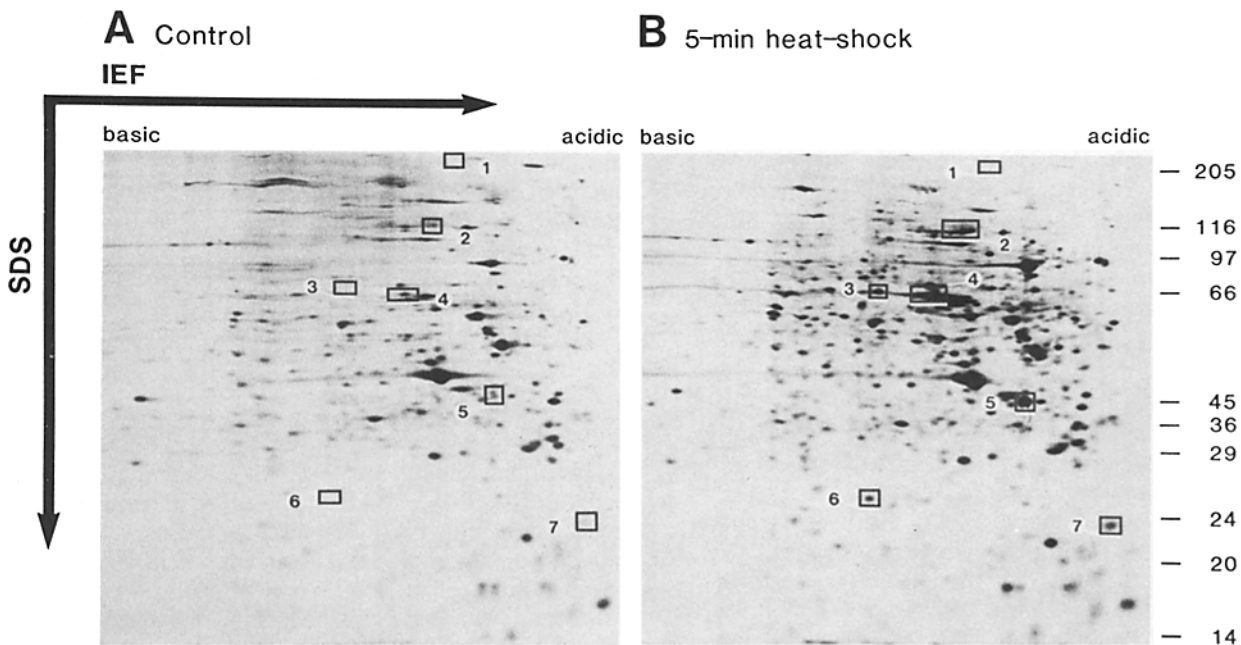
To assure that HSPs were actually synthesized after thermal stress and that RBC-mediated microinjection did not in some way affect the heat-shock response, HeLa cells were fused with RBCs, heat-shocked, and labeled with [ $^{35}\text{S}$ ]methionine 2 h later. Analysis of newly synthesized proteins from control and 5-min heat-shocked cells by two-dimensional gel electrophoresis revealed several prominent proteins which were present only in the heated cells (Fig. 2). Their molecular weights and isoelectric points presented in the legend to Fig. 2 are similar to those reported for HSPs in HeLa cells (15, 41, 44). Identical results were obtained with unfused cells, indicating that fusion of RBCs to HeLa cells does not induce or inhibit the production of HSPs.

After heat-shock, some newly synthesized HSPs might accumulate as ubiquitin conjugates. To test this possibility, HeLa cells were injected with ubiquitin, and conjugates from control and heated cells were analyzed by two-dimensional gel electrophoresis. As shown in Fig. 3, prominent ubiquitin conjugates did not co-migrate with HSPs, nor did the pattern of discrete ubiquitin conjugates change after heat-shock.

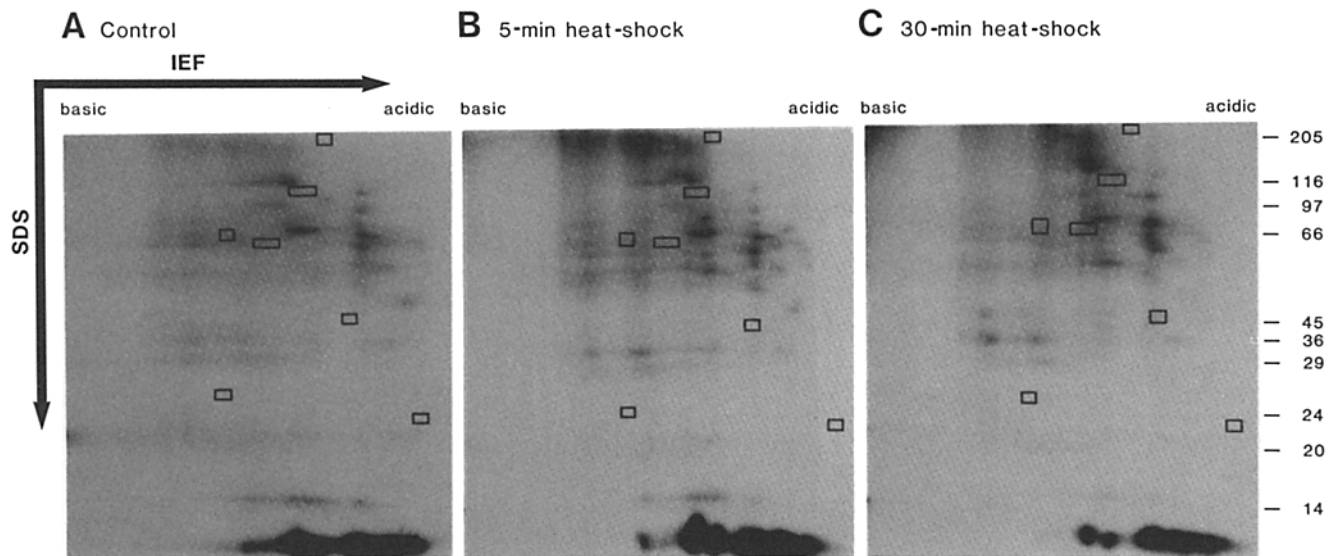


**Figure 1.** Molecular weight distribution of ubiquitin after heat-shock. (A) Autoradiogram of SDS PAGE analysis of  $^{125}\text{I}$ -ubiquitin conjugates in RBCs, heat-shocked cells, and control HeLa cells. The first lane (RBC) shows  $^{125}\text{I}$ -ubiquitin in loaded RBCs, the adjacent lane ( $\Phi$ ) is the pattern of ubiquitin conjugates immediately after fusion of the RBCs and HeLa cells, and the lane denoted Con depicts the ubiquitin conjugate pattern from injected HeLa cells after 11 h of incubation at  $37^\circ\text{C}$ . Lanes 5', 10', and 30' show the molecular weight distributions of ubiquitin conjugates in HeLa cells grown at  $37^\circ\text{C}$  for 8 h, heat-shocked at  $45^\circ\text{C}$  for the times indicated, and returned to  $37^\circ\text{C}$  for 3 h before analysis by SDS PAGE. Each lane contained 6,000 cpm of  $^{125}\text{I}$ -ubiquitin; autoradiographic exposure was 7 d. (b) Effect of protein synthesis inhibitors on the redistribution of ubiquitin after heat-shock.

HeLa cells which had been injected with ubiquitin were prepared as in A except that just before heat-shock, cycloheximide was added to a final concentration of  $100\ \mu\text{M}$ . There were 20,000 cpm of ubiquitin in each lane; autoradiographic exposure was for 2 d.



**Figure 2.** Appearance of HSPs in HeLa cells after heat-shock. HeLa cells were fused with RBCs, then given a 20-min pulse of  $^{35}\text{S}$ methionine (see Materials and Methods). Proteins extracted from these cells were then analyzed by two-dimensional PAGE and regions of the gel where HSPs appear are outlined. The proteins analyzed in Fig. 3 A were synthesized in cells grown continuously at  $37^\circ\text{C}$ . The proteins analyzed in Fig. 3 b were synthesized in cells subjected for 5 min to  $45^\circ\text{C}$  2 h before addition of  $^{35}\text{S}$ methionine. Each gel contained  $10^6$  cpm of  $^{35}\text{S}$ methionine; autoradiographic exposure was 7 d. The molecular weights and isoelectric points of the prominent HSPs are as follows: 200,000, pI 5.8; 107,000, pI 5.9; 76,000, pI 6.3; 73,000, pI 6.2; 41,000, pI 5.6; and 24,000, pI 6.4. On the gel shown above, actin migrated with an apparent molecular weight of 46,000 and an isoelectric point of 5.8; the corresponding published values are 43,000 and 5.4, respectively (17).



**Figure 3.** Electrophoretic comparison of ubiquitin conjugates and HSPs. HeLa cells injected with  $^{125}\text{I}$ -ubiquitin were either continuously grown at  $37^\circ\text{C}$  (A), or were heat-shocked at  $45^\circ\text{C}$  for 5 min (B) or for 30 min (C). Heat-shocked cells were incubated for 2 h at  $37^\circ\text{C}$ , then analyzed by two-dimensional PAGE. Autoradiographic exposure for each gel was 40 d; 23,000, 26,000, and 31,000 cpm of  $^{125}\text{I}$ -ubiquitin were analyzed on gels A-C, respectively. The regions of the gel where HSPs were observed in Fig. 2 are outlined. The molecular weights and isoelectric points for the prominent ubiquitin conjugates are as follows: 115,000, pI 6.1; 104,000, pI 5.6; 92,000, pI 5.6; 80,000, pI 5.9; 76,000, pI 5.6; 71,000, pI 5.6; 68,000, pI 5.9; 66,000, pI 5.6; 61,000, pI 5.9; 35,000, pI 6.4; and 34,000, pI 6.7.

Rather, the additional HMW conjugates that form after heat treatment are predominantly found as “smears” in the molecular weight region above 150,000 consistent with the hypothesis that ubiquitin is forming conjugates with a variety of denatured proteins (see Fig. 3 B).

Since the HSPs listed in the legend to Fig. 2 are not ubiquitin conjugates, we can eliminate the possibility that prominent HSPs arise by conjugation of ubiquitin to pre-existing proteins. This does not imply that HSPs are incapable of forming conjugates with ubiquitin, merely that the major HSPs are not ubiquitinated. If ubiquitin-HSP conjugates are present, then they should appear exclusively in heat-shocked cells, and some conjugates enriched in stressed cells do correspond in size to mono-ubiquitinated HSPs. They are observed as minor species at 86,000 (pI 5.0), 47,000 (pI 6.4), 46,000 (pI 5.7), and 32,000 (pI 5.8–6.1). Of course, these ubiquitin conjugates may simply be intermediates in the degradation of HeLa proteins denatured by heat treatment.

#### **Localization of Ubiquitin after Heat-Shock**

Some HSPs accumulate in the nucleus (43, 45), and others associate with the cytoskeleton (29) after heat-shock. To determine whether the location of ubiquitin changed after heat-shock, the intracellular distribution of injected ubiquitin in control and heat-shocked cells was determined by two methods, autoradiography of sectioned cells and extraction of cells with buffers containing 0.5% Triton X-100. It is apparent from the results presented in Table I that ubiquitin redistributes to the Triton X-100-insoluble fraction in cells that have been heat-shocked. However, autoradiography of thin sections showed no major shift of ubiquitin to either the nucleus or cytosol after heat-shock.

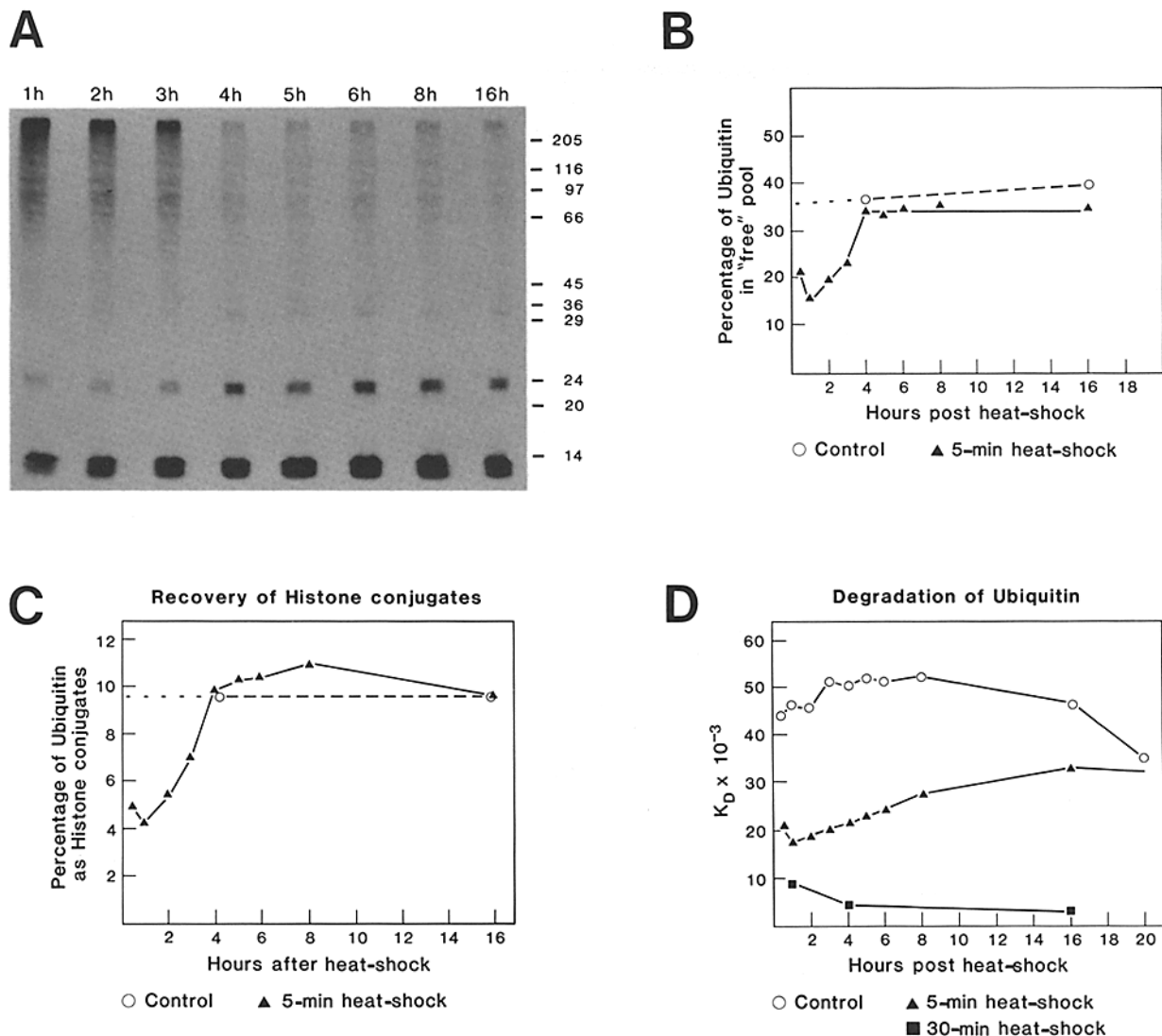
#### **Ubiquitin Stability in Heat-Shocked Cells**

Under normal culture conditions, ubiquitin is degraded in HeLa cells with a half-life <20 h (10, 48); after heat-shock,

its half-life increases substantially (Table II). In HeLa cells heat-shocked for 5 min and in those receiving a 30-min heat-shock, ubiquitin redistributes from the free pool and histone conjugates into HMW conjugates (Fig. 1). However, the fate of cells differs significantly after the two treatments. Cells heat-shocked for 5 min do not die, the degradation of ubiquitin is only transiently decreased, and the levels of free ubiquitin, histone conjugates, and HMW conjugates eventually return to control levels (Fig. 4). The recovery of a steady-state pool of free ubiquitin molecules is accompanied by reformation of histone conjugates, but the degradation of ubiquitin lags behind both events (see Fig. 4). Thus, degradation of ubiquitin does not appear to depend solely on the availability of free ubiquitin molecules, but may require reactivation of the system that degrades it. In contrast, after a 30-min heat-shock, HeLa cells never recover, and as late as 50 h after heat-shock, ubiquitin remains in HMW forms (Fig. 5). Ubiquitin is also greatly stabilized in these cells (see Table II).

#### **Proteolysis after Heat-Shock**

Under normal culture conditions an increase in proteolysis of abnormal substrates is accompanied by an increase in intracellular ubiquitin conjugates (11, 22). The levels of HMW ubiquitin conjugates are also increased after heat-shock; so one might expect an increase in proteolysis. However, pulse-chase experiments using [ $^{35}\text{S}$ ]methionine revealed a decrease in the rate of proteolysis of both short-lived and long-lived endogenous proteins after heat-shock (Fig. 6). Degradation of injected BSA, hemoglobin, and denatured hemoglobin was also reduced in heat-shocked cells (Table II and Fig. 7). However, proteolytic selectivity, per se, was not affected by heat treatment because stressed cells degraded phenylhydrazine-denatured hemoglobin faster than native hemoglobin (Fig. 7). Moreover, the data in Fig. 8 illustrate that labeled hemoglobin and labeled ubiquitin both form conjugates in



**Figure 4.** Ubiquitin pools and rate of ubiquitin degradation after heat-shock. (A) HeLa cells were injected with  $^{125}\text{I}$ -ubiquitin and heat-shocked for 5 min. After SDS PAGE analysis of samples collected at various times after heat-shock, autoradiography was performed. Each lane contained 10,000 cpm of  $^{125}\text{I}$ -ubiquitin, and the gel was exposed for 7 d. The gel in A was sliced, assayed for radioactivity, and the proportion of ubiquitin in the free pool (B) and histone conjugates (C) are shown at various times after heat-shock. In parallel, the rate of degradation of  $^{125}\text{I}$ -ubiquitin in control, 5-min, or 30-min heat-shocked cells was determined at various times after heat-shock and plotted as  $K_D$  (the first-order rate constant for degradation; see reference 30) in D.

control and heat-shocked cells. Since heat-shocked cells contain little free ubiquitin, formation of substantial amounts of ubiquitin-globin conjugates must have required mobilization of ubiquitin from pre-existing HMW forms.

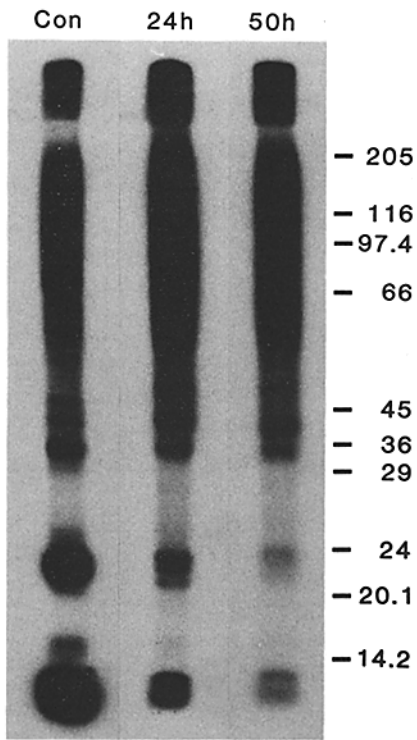
## Discussion

### Ubiquitin Conjugates and Proteolysis after Heat-Shock

We have presented data on ubiquitin metabolism in HeLa cells recovering from heat-shock. The most apparent change after heat-shock is the reduction of free and histone-conjugated ubiquitin and conversion of almost all cellular ubiquitin to HMW conjugates (Fig. 1). These characteristic responses, combined with the ability of heat-shocked cells to form ubiquitin-globin conjugates (Fig. 8), indicate that the ubiquitin-conjugating pathway remains intact even after

a lethal heat-shock. In addition to its altered distribution, ubiquitin was degraded at a markedly reduced rate in heat-shocked cells. Resistance to proteolysis is not unique to ubiquitin since endogenous proteins (Fig. 6), injected proteins (Table II), and other polypeptides (31, 46) are also stabilized in heat-shocked HeLa cells. Because reduced proteolysis cannot be attributed to impairment of the ubiquitin conjugation pathway, the activity of proteases that degrade conjugated proteins (26, 27) might be labile to heat treatment. However, denatured globin, a likely substrate for this sort of protease (11), is still degraded more rapidly than native hemoglobin in heat-shocked cells. Thus, it would seem that ubiquitin-dependent proteases retain some activity.

Heat-shock-induced redistribution of ubiquitin to the Triton X-100-insoluble fraction may also contribute to reduced rates of degradation. As shown previously, injected proteins that partition to the Triton-insoluble fraction of cells are, on the average, more stable than Triton-soluble proteins (38).



**Figure 5.** Ubiquitin pools after a 30-min heat-shock treatment. HeLa cells were injected with  $^{125}\text{I}$ -ubiquitin, heat-shocked for 30 min, and returned to  $37^\circ\text{C}$ . Proteins in cells harvested at 24 and 54 h after heat-shock were analyzed by SDS PAGE. Each lane contained 6,000 cpm of  $^{125}\text{I}$ -ubiquitin, and autoradiographic exposure was for 11 d.

Hence, it is noteworthy that the shift of ubiquitin conjugates to the Triton-insoluble fraction (Table I) coincides with the decrease in overall proteolysis.

Two other models, one based on competition of substrates

**Table II. Half-lives of Microinjected Proteins\***

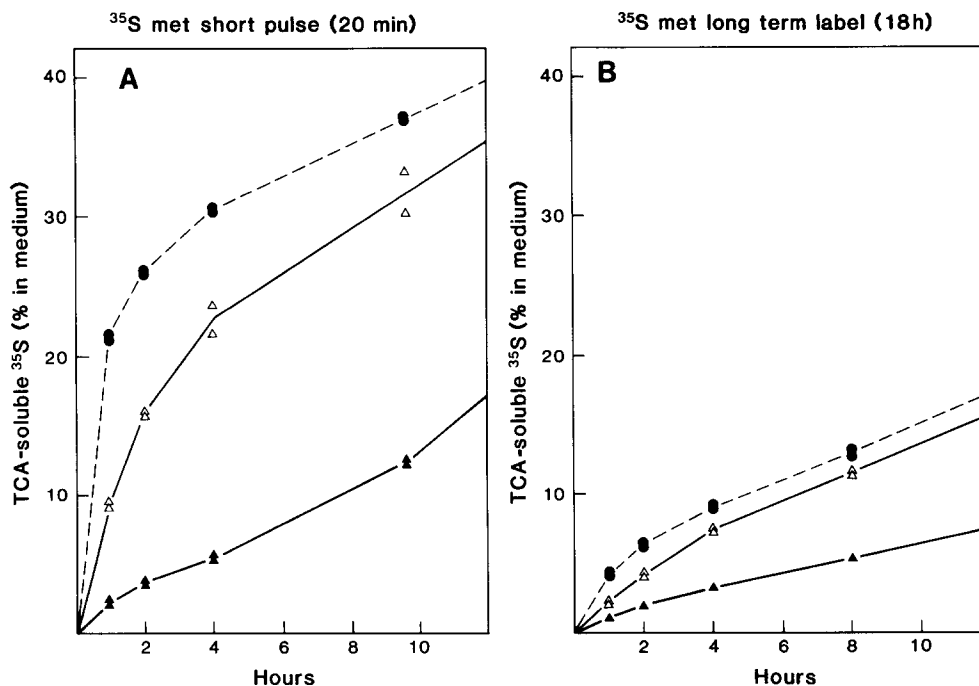
Protein	Method of radioiodination	Pretreatment of injected cells	Half-life of injected protein <i>h</i>	Increase in half-life
Hemoglobin (oxy)	BH/CT <sup>‡</sup>	None	174.5	—
		5-min hs <sup>§</sup>	175.4	1
		30-min hs	407.6	2.3
Hemoglobin (met)	BH/CT	None	71.9	—
		5-min hs	85.4	1.2
		30-min hs	151.0	2.1
Hemoglobin (apo) (phenylhydrazine denatured)	CT	None	27.4	—
		5-min hs	30.4	1.1
		30-min hs	36.1	1.3
Hemoglobin (apo) (phenylhydrazine denatured)	BH	None	24.1	—
		5-min hs	28.9	1.2
		30-min hs	30.8	1.3
BSA	CT	None	16.2	—
		5-min hs	18.2	1.1
		30-min hs	86.7	5.4
Ubiquitin	BH	None	17.6	—
		5-min hs	26.5	1.5
		30-min hs	145.2	8.4

\* The proteins listed were microinjected into HeLa cells, and their degradation was determined by the release of acid-soluble radiolabel into the medium. Half-lives were then calculated, and the stabilization in heat-shocked cells is expressed as the relative increase in half-life compared to control cells.

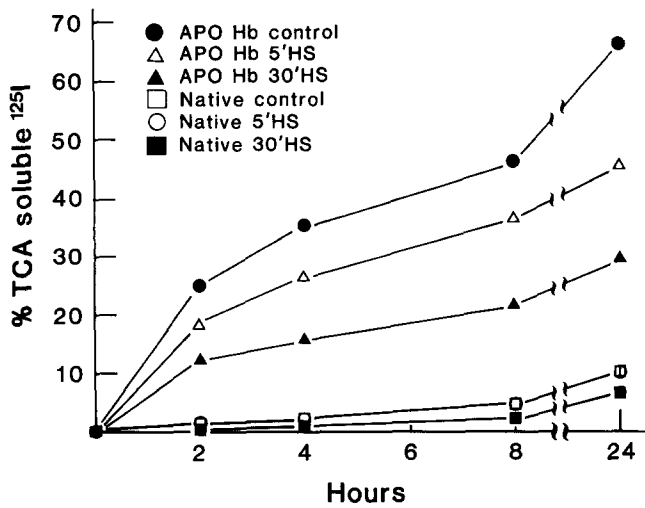
<sup>‡</sup> BH, Bolton-Hunter; CT, chloramine-T.

<sup>§</sup> hs, heat-shock.

for ubiquitination, the other based on competition for proteases, could account for the stabilization of proteins after heat-shock. Competition for limiting amounts of ubiquitin could reduce proteolysis if attachment of multiple ubiquitins were required to signal peptide bond cleavage (21). Even though there is an increase in the overall level of ubiquitin



**Figure 6.** Effect of heat-shock on proteolysis of endogenous HeLa proteins. HeLa cells were pulsed at  $37^\circ\text{C}$  with  $^{35}\text{S}$ -methionine for 20 min or for 18 h. Proteolysis of endogenous proteins was then measured in cells which had not been heat-shocked (solid circle), or from cells which had been shocked for 5 (open triangle) or 30 (solid triangles) minutes and returned to  $37^\circ\text{C}$ . The graph shows the acid-soluble radioactivity released into the media from each set of cells (33).



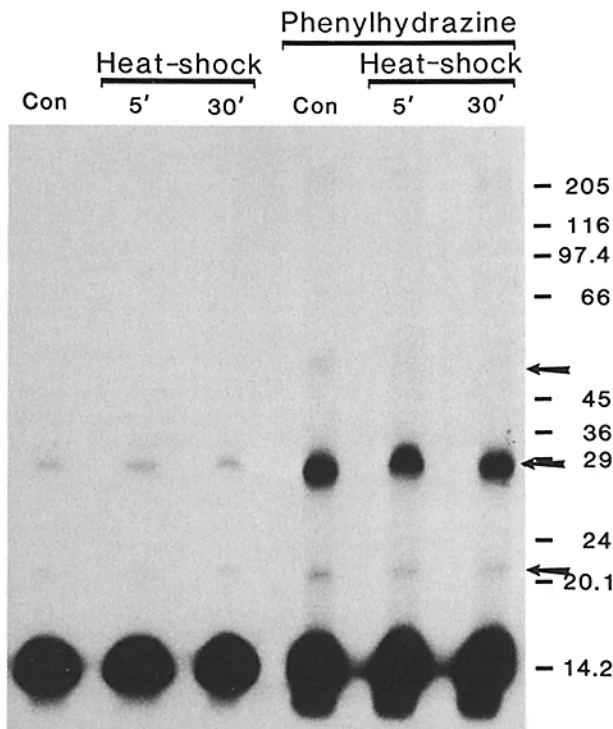
**Figure 7.** Effect of heat-shock on proteolysis of injected hemoglobin. HeLa cells were injected with  $^{125}\text{I}$ -hemoglobin, and the release of acid-soluble radiolabel into the media was measured for control cells (*open squares*), cells heat-shocked for 5 min (*open circles*), and cells heat-shocked for 30 min (*solid squares*). Some cells were also treated with phenylhydrazine to follow the degradation of denatured globin: control cells (*solid circles*), cells heat-shocked for 5 min (*open triangles*), and cells heat-shocked for 30 min (*solid triangles*).

conjugates after heat-shock, individual proteins might be insufficiently ubiquitinated to elicit proteolysis. In the second model, the accumulation of ubiquitin conjugates could saturate the protease and, by competition, stabilize proteins such as denatured globin. This proposal is consistent with both the accumulation of ubiquitin conjugates and the stabilization of some substrates. Still, if the ubiquitin pathway is responsible for most proteolysis before heat treatment, the hypothesis cannot account for the overall decrease in degradation of endogenous proteins (Fig. 6). To do so, one has to invoke the possibility of another proteolytic pathway(s) which is labile to heat. The existence of alternate pathways could explain why denatured hemoglobin is rapidly degraded after heat treatment, whereas other proteins, such as BSA and ubiquitin, are markedly stabilized, and the overall rate of proteolysis is reduced. Finally, heat treatment may affect proteolysis through changes in pH or the concentration of various ions. Reduced proteolysis or loss of  $\mu\text{H2A}$  (3) resulting from a decrease in nucleotide triphosphate pools is unlikely since ATP levels in Chinese hamster ovary cells are unaltered unless exposure to  $45^\circ\text{C}$  exceeds 45 min (9).

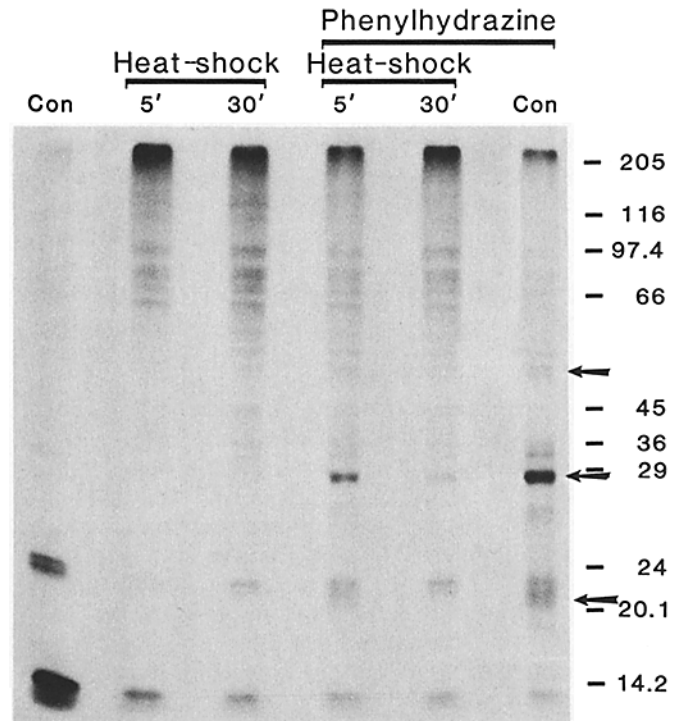
### The Stress Response and Proteolysis

In 1980, Hightower suggested that HSPs are synthesized in response to the generation of abnormal proteins and that HSPs may be involved in the degradation of these newly formed substrates (23). This hypothesis has received in-

## Hemoglobin



## Ubiquitin



**Figure 8.** Formation of ubiquitin-globin conjugates after heat-shock. HeLa cells were injected with  $^{125}\text{I}$ -hemoglobin or  $^{125}\text{I}$ -ubiquitin. Injected cells were either grown continuously at  $37^\circ\text{C}$  (control) or were heat-shocked for 5 or 30 min and returned to  $37^\circ\text{C}$ . After heat-shock, one flask from each group was treated with phenylhydrazine to denature the injected hemoglobin, and the increase in ubiquitin-globin conjugates was measured by SDS PAGE. Arrows mark bands that correspond in molecular weight to the addition of one, two, or four ubiquitin molecules to globin. There were  $\sim 8,000$  cpm of  $^{125}\text{I}$ -hemoglobin per lane in the gel on the left; autoradiographic exposure was for 14 d. Lanes in the gel on the right contained about 10,000 cpm of  $^{125}\text{I}$ -ubiquitin; exposure was for 3 d.

creasing support over the past 6 years. HSPs are synthesized in *Drosophila* cells producing mutant actins (25); HSP synthesis can be induced in oocytes after injection of denatured proteins (1); agents that stabilize proteins can inhibit certain inducers of stress response (24); and two known HSPs, *Escherichia coli* protease La and ubiquitin, are components of proteolytic pathways (6, 35). It remains to be determined whether other proteolytic pathway components are also HSPs. The small HSPs (20,000–30,000) are similar in size to E2's, the ubiquitin carrier proteins (36), and to the subunits of a large, neutral protease present in a number of tissues (14, 37, 47).

### **Proteolysis and Heat-Shock Transcription Factors**

In *E. coli*, the synthesis of protease La (the lon gene product) (4, 20) and various other HSPs (49) are under the control of the heat-shock transcription factor, htp<sub>r</sub>. Goff and Goldberg (19) proposed that the usually short-lived htp<sub>r</sub> could be stabilized after heat-shock if heat-denatured proteins competed for the protease. Stabilization of htp<sub>r</sub> would then allow synthesis of protease La, as well as other HSPs. In eukaryotic cells similar schemes involving ubiquitination of an as-yet-unidentified, heat-shock transcriptional activator have been proposed (16, 32). The hypothetical heat-shock transcription factor would be ubiquitinated under normal conditions, thereby either making it a substrate for ubiquitin-dependent proteolysis as suggested by Finley et al. (16) or inactivating it as suggested by Munro and Pelham (32). After heat-shock, this transcription factor would be stabilized by competition with other substrates for ubiquitin-dependent proteolysis (16) or activated by conversion to its non-ubiquitinated form (32). In either case, the extent of ubiquitination of a heat-shock transcription factor would determine whether HSP synthesis occurred. In fact, one could propose that ubiquitinated histones alone are sufficient to prevent transcription of heat-shock genes. Regulation of the stress response would then be sensitive to levels of abnormal proteins as suggested by Hightower (23) and could be reversed by increasing ubiquitin levels or by degrading the abnormal proteins, thereby restoring the free ubiquitin pool. Such models are consistent with the data presented in this study and can explain why ts85 cells, impaired in ubiquitin conjugation at 39°C, also synthesize HSPs at that temperature (16). However, the distribution of injected ubiquitin in HMW, histone-conjugated, and free forms changed in ts85 cells after a shift to the nonpermissive temperature of 39.5°C and resembled that seen in HeLa cells after heat-shock (Wells, R., R. Hunter, N. Carlson, and M. Rechsteiner, manuscript in preparation). If the ubiquitin conjugation in heated ts85 cells cannot be attributed to trace amounts of E1 transferred from RBCs, then decreased proteolysis in ts85 cells at nonpermissive temperature may result from these cells undergoing the stress response and not simply result from a failure in ubiquitination.

Does ubiquitination act exclusively to mark proteins for degradation? The presence of ubiquitin on histones and the lymphocyte homing receptor suggests otherwise (40, 42). Ubiquitin appears to be a multifunctional protein involved in chromatin structure, intracellular proteolysis, extracellular receptors, and the stress response. This abundant protein may serve as an intracellular barometer whose distribution among several pools regulates a variety of cellular processes.

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