Microinjection of Ubiquitin: Intracellular Distribution and Metabolism in HeLa Cells Maintained under Normal Physiological Conditions

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Abstract. Radioiodinated ubiquitin was introduced into HeLa cells by erythrocyte-mediated microinjection. Subsequent electrophoretic analyses revealed that the injected ubiquitin molecules were rapidly conjugated to HeLa proteins. At equilibrium, 10% of the injected ubiquitin was conjugated to histones and 40% was distributed among conjugates of higher molecular weight. Although the remaining ubiquitin molecules appeared to be unconjugated, the free pool of ubiquitin decreased by one-third and additional conjugates were present when electrophoresis was performed at low temperature under nonreducing conditions. Molecular weights of these labile conjugates suggest that they are ubiquitin adducts in thiolester linkage to activating enzymes. Despite the fairly rapid degradation of injected ubiquitin ($t_{1/2}$ ∼10–20 h), the size distribution of ubiquitin conjugates within interphase HeLa cells remained constant for at least 24 h after injection.

The intracellular locations of ubiquitin and ubiquitin conjugates were determined by autoradiography, by differential sedimentation of subcellular fractions in sucrose, and by extraction of injected cells with buffer containing Triton X-100. Free ubiquitin was found mostly in the cytosolic or Triton X-100–soluble fractions. As expected, histone conjugates were located predominately in the nuclear fraction and exclusively in the Triton X-100–insoluble fraction. Although high molecular weight conjugates were enriched in the Triton X-100–insoluble fraction, their size distribution was similar to that of soluble conjugates.

When injected HeLa cells were exposed to cycloheximide to inhibit protein synthesis, the size distribution of ubiquitin conjugates was similar to that found in untreated cells. Moreover, high molecular weight conjugates decreased <20% after inhibition of protein synthesis. These results indicate that most ubiquitin conjugates are not newly synthesized proteins which have been marked for destruction.

UBIQUITIN is an 8,500-mol-wt, heat-stable polypeptide found in all eukaryotic cells (19). It has been sequenced from a variety of organisms (5, 12, 44, 54, 57) and comparison of yeast to human ubiquitin reveals only three amino acid substitutions in the 76 residues that comprise the protein. Such a high degree of evolutionary conservation implies an important physiological role for ubiquitin. The molecule is known to be necessary for ATP-dependent proteolysis in reticulocyte lysates (11, 25), where its ability to form covalent conjugates to other proteins is essential for this activity (58). During the conjugation reaction, the carboxyl terminus of ubiquitin forms an isopeptide linkage to ε-amino groups of lysine residues in the ubiquitinated proteins (26).

Hershko et al. (26) proposed that the covalent attachment of ubiquitin to proteins converts them to substrates for proteolytic enzymes. This hypothesis has received increasing support during the past five years (for review see reference 14). First, hemoglobin injected into cultured mammalian cells and subsequently denatured with phenylhydrazine is rapidly degraded (24), and the concentration of globin-ubiquitin conjugates that form upon denaturation is proportional to the rate of globin degradation (8). Second, proteins that incorporate amino acid analogs are usually rapidly degraded (2, 18). When Ehrlich ascites cells are exposed to amino acid analogs, there is an increase in the intracellular concentration of ubiquitin conjugates (27). Third, the mouse cell line ts85 contains a heat-labile ubiquitin activating enzyme, E_1 (15), and the ability of these cells to degrade short-lived proteins at nonpermissive temperatures is markedly impaired (10). Fourth, *Dictyostelium* calmodulin is ubiquitinated at lysine 115 and subsequently degraded in reticulocyte lysates; bovine calmodulin is methylated at lysine 115, does not conjugate to ubiquitin, and is more stable

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in lysates (20). Finally, the marking hypothesis has received direct support by the recent demonstration that reticulocyte lysates contain a large, ATP-dependent protease that degrades lysozyme molecules conjugated to ubiquitin, but does not degrade free lysozyme (29, 30).

Proteolysis may not be the only function for ubiquitin. A sizable proportion of the histone H2A and H2B pool is ubiquitinated to form uH2A and uH2B in interphase cells (41, 55, 56). Since H2A and H2B are stable proteins (60), attachment of ubiquitin to a protein does not necessarily lead to the latter's destruction. Rather, ubiquitinated histones may affect transcription as suggested by their enrichment in the HSP 70 gene of *Drosophila* (35). It has been reported that uH2A and uH2B decrease in metaphase cells and reappear at anaphase (37, 41, 60). This transient disappearance may influence chromatin condensation (37) or cell cycle progression (61), or may serve to protect histones against proteolysis upon dissolution of the nuclear envelope (8).

Increased knowledge of the intracellular location, stability, and molecular weight distribution of ubiquitin conjugates should contribute to an eventual understanding of the roles of this highly conserved protein. Injection of radioiodinated ubiquitin into cultured cells is ideal for such an analysis. Since ubiquitin is the only labeled protein in the injected cells, formation of conjugates can readily be detected by SDS PAGE and autoradiography. Moreover, unlike immunological methods, conjugate detection does not depend on ubiquitin conformation. We report here on the behavior of ubiquitin injected into HeLa cells grown under normal culture conditions. In the following paper (6), we describe changes in ubiquitin metabolism after heat-shock.

Materials and Methods

Purification of Ubiquitin

Human ubiquitin was isolated from red blood cells (RBCs)[1] by a modification of the procedure of Wilkinson and Audhya (58). Briefly, 900 ml of packed RBCs were added slowly to 2,700 ml of water preheated to 85-90°C to heat-precipitate contaminating proteins. This solution was then filtered through Whatman No. 1 paper, ^{125}I-ubiquitin was added as a tracer, and contaminating proteins were precipitated with 40% saturated ammonium sulfate. After centrifugation at 9,200 *g* for 25 min, the supernate was taken to 85% saturation in ammonium sulfate, centrifuged as above, and the protein precipitate was dissolved in 70 ml of 10 mM sodium phosphate, 20 mM potassium chloride, pH 7.0. The sample was dialyzed against three 1-liter changes of the same buffer before chromatography on a 30-ml column of DE-52 cellulose.

Peak ubiquitin fractions, located from the position of the added ^{125}I-ubiquitin, were pooled and adsorbed to a 170-ml hydroxyapatite column preequilibrated with 5 mM sodium phosphate, pH 6.8. Ubiquitin was eluted at 50 mM sodium phosphate, pH 6.8, and then dialyzed against water before lyophilization. The lyophilized sample was dissolved in 7 ml of 0.1 M ammonium carbonate and chromatographed on a 2.5 × 20 cm column of Sephadex G-50 at 4°C. The peak fractions were pooled, dialyzed against 10 mM potassium phosphate, pH 6.0, absorbed onto a 30-ml CM-52 column, and eluted with a 25-125 mM potassium chloride gradient. The ^{125}I-ubiquitin, which eluted at 36 mM potassium chloride, was dialyzed against three 1-liter changes of water, lyophilized, and stored dessicated at 4°C.

SDS PAGE analysis after radiolabeling demonstrated that the isolated ubiquitin co-migrated with a known ubiquitin standard and was >95% pure. HPLC analysis confirmed that >95% of the isolated ubiquitin eluted as a single peak (Smith, S., and W. Gray, unpublished observation).

1. *Abbreviations used in this paper*: BH-ubiquitin, ubiquitin labeled according to the Bolton-Hunter procedure; CT-ubiquitin, chloramine-T-labeled ubiquitin; HMW, high molecular weight; RBC, red blood cell.

Radiolabeling of Ubiquitin

Ubiquitin was radiolabeled by one of three methods: reductive methylation with tritiated sodium borohydride as described by Tack et al. (53), radioiodination by the chloramine-T method (CT-ubiquitin) as described previously (8), or by a modification of the Bolton and Hunter procedure (4). In the latter method, 2.0 µl of a 1.7 mg/ml solution of Bolton-Hunter reagent in dioxane was mixed with 2 mCi (20 µl) of Na^{125}I in the presence of 10 µl of chloramine-T at 1 mg/ml in 0.15 M sodium phosphate buffer, pH 7.5. The reaction was quickly terminated by addition of 5 µl of a 2-mg/ml solution of sodium meta-bisulfite in 0.15 M sodium phosphate, pH 7.5. The radioiodinated Bolton-Hunter reagent was then coupled to ubiquitin by addition of 10 µl of a 3.5 mg/ml solution of ubiquitin in 0.15 M sodium borate, pH 8.5. After 30 min, the labeled ubiquitin (BH-ubiquitin) was separated from unreacted iodine and iodinated Bolton-Hunter reagent by passage through a 3-ml column of Sephadex G-25 equilibrated with 10 mM Tris, pH 7.5. Specific activities ranged from 10-20 µCi/µg for BH-ubiquitin and 0.5 to 0.9 µCi/µg for CT-ubiquitin.

3 mol of Bolton-Hunter reagent were added for each ubiquitin molecule, and typical incorporation of ^{125}I was ~33%, or ~1 Bolton-Hunter group per molecule of ubiquitin. Because the incorporation of each 3(4-hydroxyphenyl) propionamide group removes one positive charge from the protein, several isoelectric forms of ubiquitin were observed by both acid-urea and two-dimensional gel analysis (6).

CT-ubiquitin is reported to stimulate ATP-dependent proteolysis differently than native ubiquitin (59). However, the intracellular stability of ubiquitin (see Fig. 1) and the molecular weight distribution of ubiquitin conjugates were similar after injection of either CT- or BH-ubiquitin (data not shown). Because of its 10-fold higher specific activity, BH-ubiquitin was used for most experiments.

Cell Culture and RBC-mediated Microinjection

HeLa cells were maintained at 37°C in Ham's F-12 media (23) supplemented with 100 U/ml of penicillin, streptomycin, and 5% calf serum. Radiolabeled ubiquitin was introduced into the cells by RBC-mediated microinjection as described previously (51). Typically 30% of the labeled ubiquitin was retained in the RBC during loading, and 1.0% of the loaded BH-ubiquitin was transferred to the HeLa cells.

Preparation of Cell Extracts for Gel Electrophoresis

Interphase cells were harvested with 0.1% trypsin in Mg^{++}/Ca^{++}-free saline, rinsed twice with cold PBS, and dissolved in 2× SDS sample buffer (1× SDS sample buffer = 0.4 M β-mercaptoethanol, 10% glycerol, 3% SDS, and 62.5 mM Tris, pH 6.8) (34). Metaphase cells, isolated by mitotic shake-off 24 h post-injection (13), were washed two times with ice-cold PBS before dissolving in SDS sample buffer. All samples were stored frozen at −70°C before electrophoresis (34). For analysis on nonreducing gels, cell pellets were first extracted for 30 min on ice with Triton X-100 extraction buffer (16) containing 100 µg/ml DNase 1, then dissolved in 3× SDS sample buffer without 2-mercaptoethanol and immediately analyzed by SDS PAGE at 4°C. Samples for acid-urea gel analyses were prepared by extracting frozen cell pellets with 0.2 M sulfuric acid.

Cell Fractionation

Cell fractionations were performed by differential sedimentation in sucrose as described (3), or by extraction of cells with buffer containing 0.5% Triton X-100 (16) and 5 mM *N*-ethylmaleimide to inhibit isopeptidase activity. Cells were removed from the monolayer by trypsinization in 0.25 M sucrose, 1 mM EDTA, and disrupted in a Dounce homogenizer (65 strokes); cell breakage was >90%. Nuclear pellets were obtained by centrifugation at 2,000 *g* for 10 min. Cells extracted with Triton X-100 were centrifuged for 10 min at 15,600 *g* in an Eppendorf microcentrifuge to produce particulate and soluble fractions. Fractions analyzed by SDS PAGE were dissolved in 2× SDS sample buffer or were mixed with equal volumes of 4× SDS sample buffer and frozen at −70°C.

Autoradiography

HeLa cells injected with BH-ubiquitin were fixed in Karnovsky's solution (32), dehydrated in ethanol, embedded in Medcast plastic resin, and prepared for autoradiography as described previously (40).

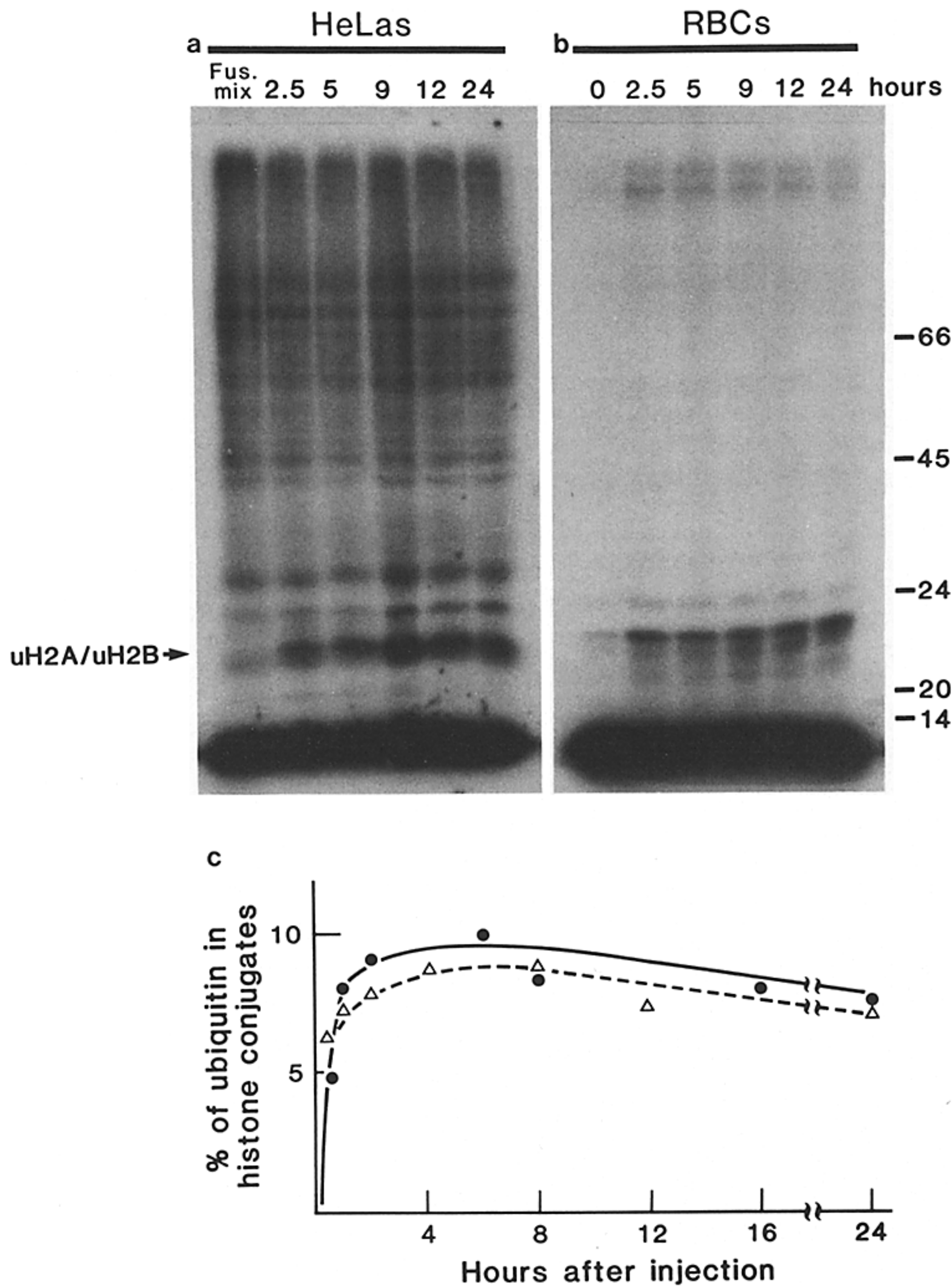

Figure 1. Fate of ubiquitin injected into HeLa cells. (*a*) Molecular weight distribution of ^{125}I-ubiquitin in injected cells. HeLa cells were fused with ubiquitin-loaded RBCs and samples obtained from the injected cells (either immediately after fusion [*Fus. mix*] or at the various times indicated above each lane) were analyzed by electrophoresis on 10% acrylamide gels. (*b*) Molecular weight distribution of ^{125}I-ubiquitin in loaded RBCs. RBCs were loaded with ^{125}I-ubiquitin and incubated at 37°C in culture medium. The fate of ubiquitin within RBCs was determined by SDS PAGE analysis and autoradiography for samples removed at the times shown. Each lane contained 20,000 cpm, and the gels were exposed for 3.5 d. Lanes marked 0 and 24 h in *b* were cut into 5-mm sections and the radioisotope in each slice was quantitated; free ubiquitin comprised 90 and 80%, respectively, of the total cpm in these lanes. (*c*) Formation of ubiquitin–histone conjugates from microinjected ubiquitin. HeLa cells were injected with ^{125}I-ubiquitin and samples taken at various times post-injection were analyzed by SDS PAGE. Gels were sliced into 5-mm segments and the amount of ^{125}I in the bands corresponding to uH2A and uH2B was determined by gamma spectroscopy. Because all of the injected HeLa cells did not attach to the culture dish within the first 2 h after fusion, unfused RBCs could not be removed by rinsing. Corrections for RBC contamination at early times were made by lysing residual RBCs in 0.83% NH_4Cl (31) or by estimating the contamination from the reduced rate of ubiquitin degradation. The corrected value is denoted by a dashed line.

Polyacrylamide Gel Electrophoresis

Proteins were analyzed by SDS PAGE (10% acrylamide) (34) or acid–urea PAGE (45). Molecular weight standards, obtained from Sigma Chemical Co. (St. Louis, MO), consisted of rabbit myosin (205,000), β-galactosidase (116,000), rabbit phosphorylase B (97,400), BSA (66,000), egg albumin (45,000), rabbit glyceraldehyde 3-phosphate dehydrogenase (36,000), bovine carbonic anhydrase (29,000), PMSF-treated trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,200). Acid-extracted samples were brought to a final concentration of 8 M urea, 20 mM Tris, and 7% 2-mercaptoethanol before being applied to acid urea gels. Standards for these gels were histones H1, H2A, H2B, H3, and H4 (a gift from L. Wu, University of Utah).

Some gels were stained in 0.2% Coomassie Brilliant Blue in 22.5% methanol, 7.5% acetic acid for 1 h, and subsequently destained in methanol/acetic acid alone. Gels were then dried onto Whatman 3MM paper (Whatman Inc., Clifton, NJ) and exposed at −70°C to either Kodak XAR or BB-1 X-ray film with DuPont Lightning plus intensifier screens. To pre-

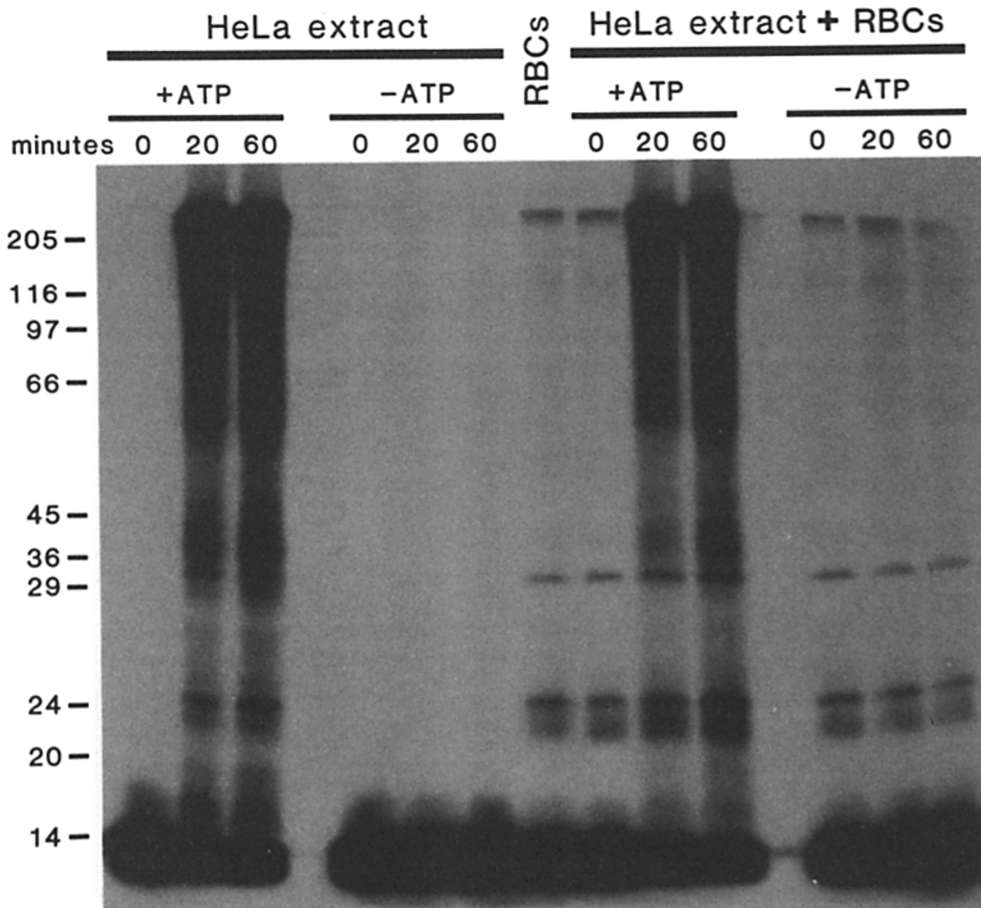

Figure 2. Ubiquitin conjugation in HeLa extracts. Extracts were prepared from uninjected HeLa cells (14) and incubated with 1.5×10^6 cpm of ^{125}I-ubiquitin in a final volume of 100 μl. Labeled ubiquitin was either added to the extract directly from the iodination mixture (*HeLa extract*), or from ubiquitin-loaded RBCs lysed directly in the extract (*HeLa extract + RBCs*). These samples were incubated in the presence of an ATP regeneration system (*+ATP*) or ATP depletion system (*−ATP*) as described (7). SDS PAGE analysis was conducted on samples removed immediately after mixing (0 time) or after a 20- or 60-min incubation at 37°C.

vent loss of radiolabeled ubiquitin in experiments involving direct quantitation, unstained gels were dried onto Whatman 3 MM paper, and lanes were cut into 5-mm pieces. Each slice was then counted in a gamma counter (model 4000; Beckman Instruments, Inc., Palo Alto, CA).

Measurement of Proteolysis

The release of acid-soluble and acid-insoluble radioactivity from injected HeLa cells was measured as described (40). Paper chromatography confirmed that >90% of the released TCA-soluble radioactivity was iodotyrosine (CT-ubiquitin) or iodo-Bolton–Hunter lysine (BH-ubiquitin) (50). Rates of proteolysis were calculated by the method of McGarry et al. (40) or Neff et al. (43).

Materials

Calf sera were obtained from Flow Laboratories, Inc. (McLean, VA) or Sterile Systems, Inc. (Logan, UT); Ham's F-12 medium was obtained from Gibco (Santa Clara, CA). Radiochemicals were obtained from Amersham Corp. (Arlington Heights, IL). Bis-acrylamide was obtained from Eastman Kodak Co. (Rochester, NY). Acrylamide, chloramine-T, sodium metabisulfite, and ammonium sulfate were from J. T. Baker Chemical Co. (Phillipsburg, NJ). Bolton–Hunter reagent was obtained from Calbiochem-Behring Corp. (San Diego, CA) under the commercial name of Tagit. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

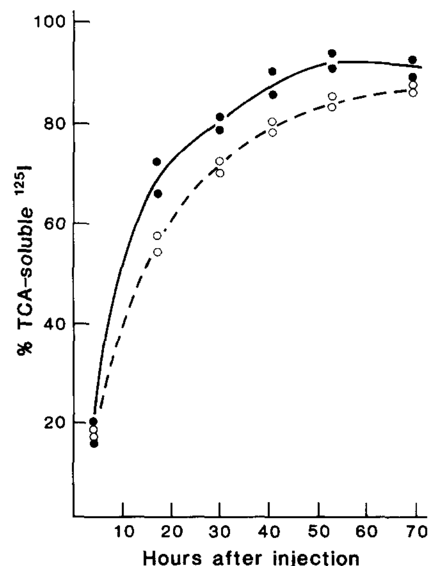

Figure 3. Degradation of injected ubiquitin. HeLa cells were injected with ubiquitin labeled with ^{125}I either by chloramine-T oxidation or by reaction with ^{125}I-Bolton–Hunter reagent (see Materials and Methods). Release of acid-soluble ^{125}I into the medium was then determined, and rates of proteolysis were calculated by the method of Neff et al. (43). (*Solid circles*) CT-ubiquitin; (*open circles*) BH-ubiquitin.

Results

Ubiquitin Conjugation and Degradation in Injected HeLa Cells

The fate of ubiquitin after injection into HeLa cells is shown in the electrophoretic pattern presented in Fig. 1. Imme-

diately after injection, ubiquitin formed higher molecular weight conjugates (see lane labeled *Fus. mix*, Fig. 1 *a*). Except for the slower appearance of ubiquitinated histones, there was little change in the size distribution of conjugates thereafter (Fig. 1 *a*, lanes *2.5–24 hours* and Fig. 1 *c*). Small amounts of ubiquitin conjugates were present in loaded RBCs before injection and incubation of loaded RBCs in culture media for up to 24 h converted an additional 10% of the loaded ubiquitin to higher molecular weight forms (Fig. 1 *b*). These data and data demonstrating the remarkable stability of ubiquitin in RBCs ($t_{1/2} > 30$ d) indicate that ubiquitin is not altered in RBCs, but these results do not rule out the possibility that formation of ubiquitin conjugates in recipient HeLa cells is due to components transferred from the RBCs. Earlier reports that ubiquitin introduced into HeLa cells by osmotic lysis of pinosomes was capable of forming conjugates suggested that HeLa cells contain an intact conjugation pathway (8). This was confirmed by analysis of ubiquitin conjugation in vitro. Extracts prepared from uninjected HeLa cells were capable of conjugating ubiquitin to a variety of endogenous proteins, and addition of RBC lysate to this extract contributed no additional conjugating activity (Fig. 2).

From pulse–chase experiments, Wu et al. (60) reported that native ubiquitin is lost from Chinese hamster ovary cells with a half-life of 9 h. In close agreement with their results, we observed that ubiquitin labeled by the chloramine-T procedure, the Bolton–Hunter procedure, or reductive methylation, and then injected into HeLa cells was lost from the cells with half-lives of 11, 13, or 16 h, respectively. Not all loss of ubiquitin was due to its degradation; some of the radiolabel was released in acid-insoluble form (data not shown). The release of acid-soluble radiolabel from HeLa cells injected with either CT- or BH-labeled ubiquitin (presented graphically in Fig. 3) showed that the two molecules were degraded at equivalent rates ($t_{1/2} < 20$ h).

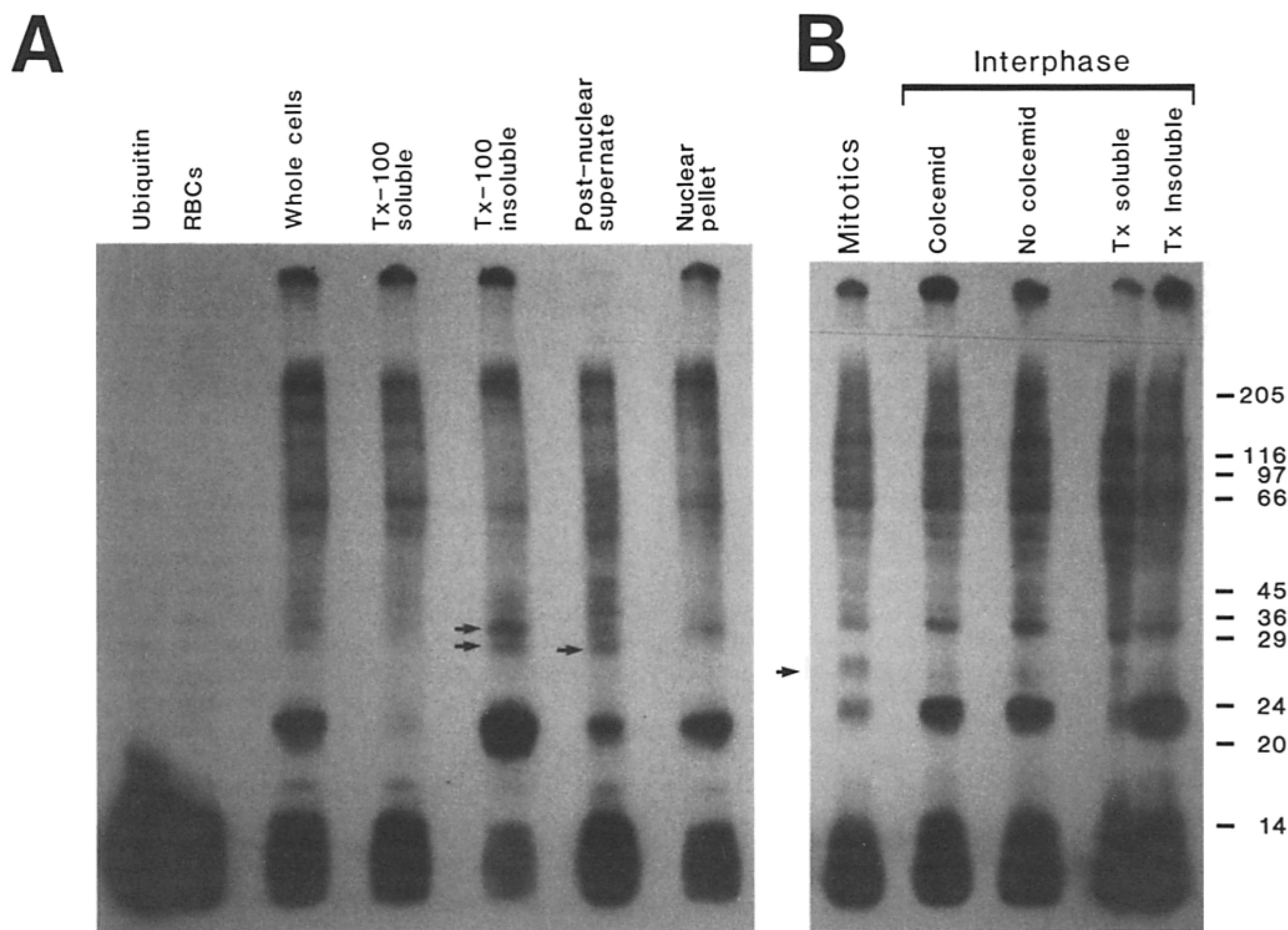

Figure 4. Distribution of ^{125}I-ubiquitin and ^{125}I-ubiquitin conjugates in injected HeLa cells. (*A*) Size distribution of ubiquitin conjugates at interphase. 12 h after injection with ^{125}I-ubiquitin, HeLa cells were extracted in buffer containing 0.5% Triton X-100 or fractionated in 0.25 M sucrose (see Materials and Methods). SDS PAGE followed by autoradiography was then used to determine the distribution of free ubiquitin and ubiquitin conjugates in Triton X-100–soluble and –insoluble fractions or in the postnuclear supernate and the nuclear pellet. Each lane contained 10,000 cpm; autoradiographic exposure was for 5 d. (*B*) Size distribution of ubiquitin conjugates in mitotic cells. HeLa cells were injected with ^{125}I-ubiquitin, and 24 h later, colcemid was added to a final concentration of 0.1 μg/ml. After 4 h, mitotic cells were isolated by shake-off and interphase cells were collected by trypsinization. SDS PAGE analysis and autoradiography were performed. Each lane contained 10,000 cpm and autoradiographic exposure was for 8 d. The mitotic index was >85% for colcemid-arrested cells. The arrow identifies a 26,000-mol-wt conjugate unique to mitotic cells.

The Intracellular Location of Ubiquitin and Ubiquitin Conjugates

For convenience, we consider injected ubiquitin to be present in three metabolic compartments or pools: the free pool, histone conjugates, and high molecular weight (HMW) conjugates. From eight independent measurements on interphase HeLa cells, the partitioning of ubiquitin among these three compartments was 42% HMW conjugates, 10% histone conjugates, and 48% in the free pool; the standard deviation for all values was <3%.

Autoradiography on thin sections of HeLa cells injected with ubiquitin revealed similar grain densities over nucleus and cytoplasm. However, autoradiography may not distinguish between free and conjugated ubiquitin. To determine the subcellular distribution of components of the three metabolic pools, injected HeLa cells were extracted with buffer containing Triton X-100 or fractionated in sucrose (see Materials and Methods). The crude nuclear and cytosolic fractions were analyzed by SDS PAGE (Fig. 4 *A*) and the distribution of ubiquitin among the various pools was determined for each fraction (Table I). At 12 h post-injection, free ubiquitin was found mainly in the Triton X-100–soluble or in the postnuclear supernate obtained after homogenization in sucrose. Ubiquitin-histone adducts were present exclusively in the Triton X-100–insoluble fraction and greatly enriched in nuclear pellets. Although HMW conjugates appeared in all fractions, several differences in their size distribution were observed between Triton X-100–soluble and –insoluble fractions (arrows in Fig. 4 *A*). Similar distributions of radiolabel were found when cells were fractionated at 4 and 53 h post-fusion.

To examine ubiquitin pools at mitosis, HeLa cells were injected with BH-ubiquitin and mitotic cells were isolated 24 h later. Both metaphase-arrested cells and interphase cells were analyzed by SDS PAGE (Fig. 4 *B*) or acid–urea PAGE (not shown), and in three experiments, we consistently observed the following differences: (*a*) ubiquitin–histone conjugates were enriched by more than twofold in interphase cells (2.4-fold by SDS PAGE and 2.1-fold by acid–urea PAGE); (*b*) mitotic cells contained a unique 26,000-mol-wt ubiquitin conjugate; and (*c*) mitotic cells contained slightly more (1.3-fold) free ubiquitin. No more than half of the residual ubiquitin–histone conjugates observed in mitotic cells could be attributed to contamination from interphase cells since the mitotic index was >85% for all preparations of mitotic cells.

Table I. Localization of Ubiquitin and Ubiquitin Conjugates

Fractionation procedure	Location	Distribution of ubiquitin and ubiquitin conjugates		
		Conjugates	Free ubiquitin	Total
		%	%	%
Homogenization in 0.25 M sucrose	Postnuclear supernate	18	37	55
	Nuclear pellet	29*	16	45
	Total	47	53	100
Extraction in 0.5% Triton X-100	Soluble	29	45	74
	Insoluble	21‡	5	26
	Total	50	50	100
Direct solubilization in SDS (whole cells)	Total	51§	49	100

Lanes from the gel in Fig. 5 were cut into 5-mm segments, and gamma spectroscopy was used to determine the fraction of free and conjugated ^{125}I-ubiquitin.

* Of these conjugates, 22% were histone conjugates. This represents 6.5% of the total ubiquitin and suggests isopeptidase activity in sucrose fractionated nuclei since cells solubilized in Triton X-100 or SDS had a higher proportion of histone conjugates (see below).

‡ Of these conjugates, 47% were histone conjugates; this represents 9.9% of the total ubiquitin.

§ Of these conjugates, 22% were histone conjugates; this represents 11.1% of the total ubiquitin.

Characterization of the Three Ubiquitin Pools

The Free Pool. The free ubiquitin pool might consist of active but unconjugated molecules or of damaged molecules incapable of conjugation. The presence of inactive ubiquitin is a distinct possibility since the COOH-terminal glycine residues can be easily removed from this protein by trypsin (58), or an unidentified protease possibly of lysosomal origin (22). To estimate the proportion of injected ubiquitin molecules capable of conjugation, HeLa cells injected with BH-ubiquitin were treated with phenylhydrazine to denature the co-injected hemoglobin. Confirming earlier results (8), we found that >85% of the injected ubiquitin appeared as conjugates, most with molecular weights expected for globin-ubiquitin adducts (Fig. 5). These results indicate that at least 85% of the injected ubiquitin was active. In the following paper (6), we report even greater conversion of ubiquitin to HMW forms after heat-shock. Thus, the substantial size of the free pool in HeLa cells maintained under normal growth conditions cannot be attributed to the presence of inactive ubiquitin molecules.

To determine what fraction of the free ubiquitin pool may have arisen by release from conjugates during SDS PAGE analysis (e.g., ubiquitin in thiol ester linkage to activating enzymes [9, 28, 46]), proteins from injected cells were electrophoresed at low temperature under nonreducing conditions. Fig. 6 shows that additional conjugates were present in samples analyzed in this manner. The most prominent of these had a molecular weight of 110,000, the size expected for a ubiquitin-E_1 thiol conjugate (9). Unique conjugates at 30,000 and 38,000 were also observed under nonreducing conditions, and they may represent ubiquitin-E_2 thiol adducts. The existence of other ubiquitin-E_2 thiol conjugates is suggested by a twofold increase in radioactivity in the 20,000–24,000-mol-wt region of the gel under nonreducing conditions. Thus, using mild preparative procedures, the free pool decreased by one-third suggesting that as much as 15% of the injected ubiquitin associates with HeLa proteins in complexes labile to reduction and/or boiling. Over 80% of these labile conjugates have molecular weights consistent with those expected for complexes between ubiquitin and the E_1 or E_2 components of the activation pathway (46).

Histone Conjugates. The most prominent ubiquitin conjugates in HeLa cells, a doublet with a molecular weight of ~22,000 on SDS PAGE (Fig. 1 *a*), are ubiquitin–histone adducts by the following criteria: (*a*) they appear only in nuclear fractions (Fig. 4) and form more slowly than HMW conjugates (Fig. 1 *c*); (*b*) their molecular weights are consis-

tent with those expected for uH2A and uH2B; (*c*) they comigrate with a 22,000-mol-wt molecule formed from ^{125}I-H2A in rabbit reticulocyte lysates; and (*d*) they decrease significantly at mitosis (Fig. 4 *B*). As already noted, ubiquitin–histone conjugates form more slowly than HMW conjugates (Fig. 1 *c*). This is expected since in vivo studies show that uH2A becomes labeled by exchange of ubiquitin with an apparent half-life of 90 min (52).

HMW Conjugate Pool. Of the three intracellular ubiquitin pools, the HMW conjugate pool is the most diverse, containing conjugates that vary on SDS PAGE from 25,000 to greater than 250,000 (see Fig. 4). These conjugates did not dissociate from even larger soluble complexes since the majority of the injected ubiquitin sediments on sucrose density gradients as molecules with weights less than 250,000 (Fig. 7).

The physiological significance of the HMW ubiquitin conjugates is not clear. Denatured hemoglobin and abnormal proteins form ubiquitin conjugates and are rapidly degraded (8, 27). If the major role of ubiquitin is to mark proteins for proteolysis, then the sizable fraction of newly synthesized proteins that are rapidly degraded (47) might be expected to form conjugates with ubiquitin. This hypothesis, which predicts a decline in HMW conjugates upon inhibition of protein synthesis, was tested by incubating injected cells in media containing cycloheximide and comparing conjugate pools to those of control cells (Fig. 8). Even 8 h after blocking protein synthesis, none of the HMW conjugate bands disappeared. The HMW pool did, however, decrease by 20% in cycloheximide-treated cells, the decrease occurring principally in conjugates with molecular weights larger than 66,000. These results suggest that most HMW conjugates are not proteolytic intermediates of newly synthesized, rapidly degraded proteins.

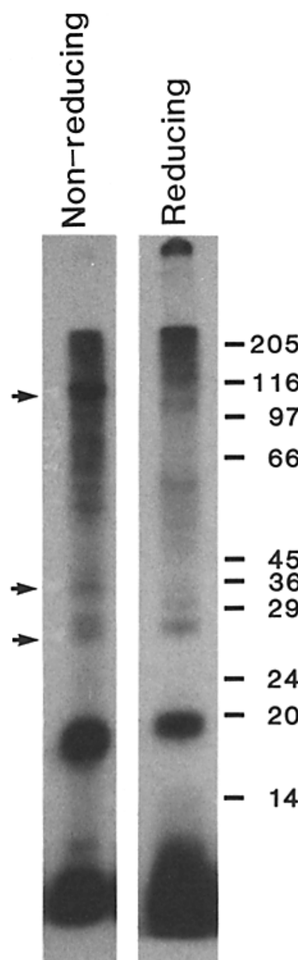

Figure 6. Analysis of ubiquitin conjugates under nonreducing conditions. HeLa cells were injected with ^{125}I-ubiquitin and 6 h later cells were either dissolved in SDS sample buffer containing 2-mercaptoethanol and boiled (*Reducing*) or extracted with Triton X-100 buffer containing DNase I and then dissolved in SDS sample buffer lacking 2-mercaptoethanol (*Non-reducing*). SDS PAGE analysis was conducted at 4°C to preserve thiol esters. Each lane contained 15,000 cpm and autoradiographic exposure was for 3 d. After autoradiography, the gel was sliced into 5-mm pieces, and the distribution of radiolabel was determined by direct counting. Prominent bands apparent under nonreducing conditions are denoted by arrows.

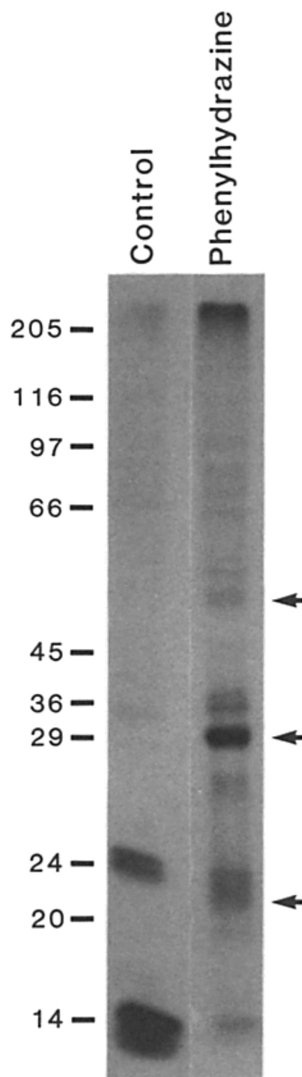

Figure 5. Ubiquitin-globin conjugates in HeLa cells. HeLa cells were injected with ^{125}I-ubiquitin, and at 12 h post-injection phenylhydrazine was added to a final concentration of 350 μM. 90 min later, treated and control cells were dissolved in SDS sample buffer, and their intracellular proteins were analyzed by SDS PAGE followed by autoradiography. Quantitation by gel slicing revealed that only 13% of the injected ubiquitin remained in the free pool after phenylhydrazine administration. Ubiquitin conjugates with molecular weights corresponding to the addition of one, two, or four ubiquitin molecules to globin were prominent in cells exposed to phenylhydrazine and are marked by arrows.

Discussion

In this study, we have presented data on the distribution and metabolism of injected ubiquitin. Implicit in our analysis is the assumption that the behavior of the injected molecule mirrors that of endogenous ubiquitin. A number of observations support this supposition. Injected ubiquitin formed conjugates to HeLa proteins including H2A and H2B, and nearly all of the injected molecules were capable of forming such conjugates (Fig. 5). Also, the data in Fig. 3 show that injected ubiquitin was degraded at a rate similar to that reported for the endogenous molecule (60). A number of other studies also suggest that injected, labeled proteins can

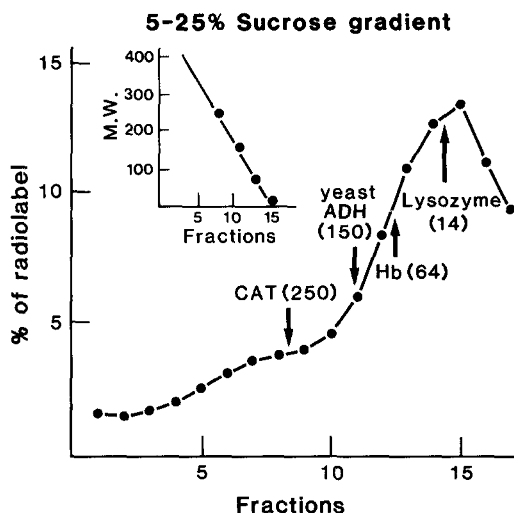

Figure 7. Sedimentation analysis of injected ubiquitin. HeLa cells were injected with ^{125}I-ubiquitin and extracted 8 h later with buffer containing Triton X-100. The soluble fraction was diluted 4:1 and *N*-ethylmaleimide was added to a final concentration of 5 mM to inhibit isopeptidases. A portion of this extract (300 μl) was then centrifuged on a 5-ml, 5-25% sucrose gradient, for 15 h at 29,000 *g* in a Beckman SW39 rotor. The gradient was collected from the bottom in 15 equal fractions, and the radioactivity in each fraction was determined. Molecular weight markers were catalase (*CAT*), 250,000; yeast alcohol dehydrogenase (*ADH*), 150,000; hemoglobin (*Hb*), 64,000; and lysozyme, 14,000. Their positions were identified as described (30).

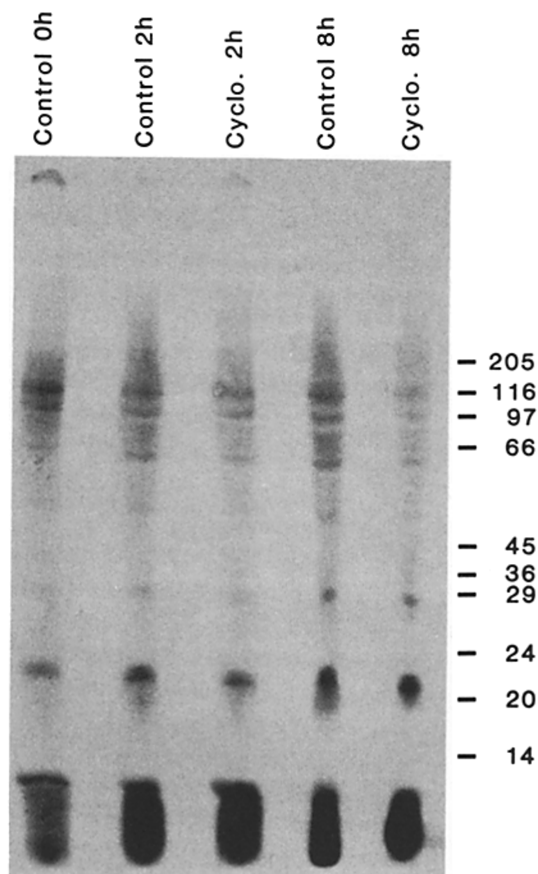

Figure 8. Fate of ubiquitin conjugates after inhibition of protein synthesis. HeLa cells were injected with ^{125}I-ubiquitin, and 5 h later medium containing 100 μM cycloheximide was added. SDS PAGE analyses of cellular proteins were performed at 2 and 8 h after addition of cycloheximide (*Cyclo*) and on cells in normal growth medium (*Control*). After SDS PAGE, autoradiography was performed with the gels being exposed for 2.5 d. Each lane contained 10,000 cpm.

serve as legitimate probes of the behavior of endogenous proteins. Examples include the accumulation of injected muscle and cytoskeletal proteins in the appropriate subcellular substructures (17, 33), the nuclear accumulation of chromosomal proteins HMG1 and HMG2 after RBC-mediated microinjection (49), and the observation that half-lives for many injected proteins closely approximate those obtained by in vivo labeling (39, 50).

Even if the injected ubiquitin molecules behave normally, they could shift the apparent distribution of ubiquitin among conjugates if they significantly altered the size of the endogenous pool. About 10^5 labeled ubiquitin molecules are transferred from each loaded RBC, and the following calculation shows this to be <0.1% of the endogenous pool. The numbers of ubiquitin and H2A molecules are roughly equal since 10% of the intracellular ubiquitin is conjugated to H2A and vice versa (55). Based on a DNA content of 17 pg per cell (36), we estimate that a HeLa cell contains 1.5×10^8 copies of H2A, hence an equivalent number of ubiquitin molecules. This value is similar to estimates by Haas and Bright (21) based on immunological methods that nucleated cells contain between 0.8 and 1.8×10^8 ubiquitin molecules. Moreover, according to Haas and Bright, an RBC contains $\sim 3 \times 10^6$ ubiquitin molecules (21). Therefore, the total contribution of labeled and unlabeled ubiquitin from RBC-mediated injection is <2% of the amount already present in a HeLa cell.

Knowing the number of ubiquitin molecules per cell, we can estimate minimal levels of various "activating" enzymes if we assume that the bands seen only under nonreducing conditions are thiol ester adducts of ubiquitin to components of the conjugation pathway. About 3% of the total ubiquitin migrates as a putative ubiquitin-E_1 complex (Fig. 6) corresponding to 4×10^6 copies of E_1 per cell. Some of the individual E_2s appear to be less abundant since the putative thiol ester conjugates migrating at 30,000 and 38,000 comprise only 1.3% and 0.3%, respectively, of the total ubiquitin injected. Other ubiquitin-E_2 complexes, which might be expected at 21,000, 25,000, and 28,000 (46) were either not present or were masked by co-migration with histone conjugates. However, an additional 10% of the injected ubiquitin migrated between 20,000 and 24,000 under nonreducing conditions, so smaller E_2 carrier proteins may be even more abundant than E_1.

Our results indicate that under normal culture conditions, 40% of the ubiquitin molecules in HeLa cells are present as HMW conjugates, 10% as histone conjugates, 15% as adducts labile to boiling and reduction, and the remaining 35% as free ubiquitin. The latter value is in agreement with findings of Haas and Bright (21) who reported that 40 and 26% of the ubiquitin is unconjugated in human IMR-90 fibroblasts and monkey CV-1 cells, respectively. In partial agreement with reports that uH2A and uH2B are absent in metaphase cells (37, 41, 60), we observed a clear reduction

in these conjugates at mitosis (Fig. 4 *B*). However, residual ubiquitin–histone conjugates were present that could not be attributed to contaminating interphase cells. Perhaps some colcemid-arrested mitotic cells had partially entered anaphase and re-ubiquitinated H2A. It is also possible that BH-ubiquitin is more resistant to isopeptidase activity (1, 38) and radioiodinated ubiquitin molecules remain attached to histones at metaphase when native ubiquitin is stripped. Finally, in contrast to Chinese hamster ovary cells or *Physarum*, HeLa cells may resemble rat hepatoma cells by retaining residual histone conjugates at metaphase (48).

Besides providing information on the partitioning of ubiquitin at equilibrium, microinjection reveals dynamic aspects of ubiquitin metabolism. HMW conjugates must be generated very rapidly because they are fully labeled within the 30 min required for fusion (see Fig. 1 *a*). The slower formation of H2A conjugates from injected ubiquitin (Fig. 1 *c*) agrees with in vivo labeling experiments showing that equilibration of newly synthesized ubiquitin with uH2A takes 1–2 h (52). The pattern of ubiquitin conjugates remained unchanged during the 30-h period after injection. This indicates that stable ubiquitin conjugates are rare or absent in HeLa cells since the concentration of such conjugates would have been expected to increase with time. The apparent absence of "stable" conjugates and the rapid redistribution of ubiquitin to denatured globin (Fig. 5) implies that most, if not all, ubiquitin molecules are in dynamic equilibrium with HeLa proteins.

Except for uH2A and uH2B, the identity of ubiquitin conjugates observed by SDS PAGE is unknown. Only 20% of the HMW conjugate pool consists of potential intermediates in the degradation of proteins with short half-lives. The remaining conjugates may be stable proteins marked for destruction or alternatively, ubiquitin conjugation may serve other functions. Several prominent conjugates, always seen between 50,000 and 130,000 and at 30,000 (cf. Fig. 4 *B* and 8), could be proteins whose activity is regulated by ubiquitin. Munro and Pelham (42) propose that a putative heat-shock transcription factor might be inactivated by ubiquitination and that ubiquitin removal activates this factor after heat-shock. The rapid depletion of free ubiquitin upon heat-shock is consistent with this proposal (see the following paper [6]). Finally, the unique 26,000-mol-wt mitotic conjugate (Fig. 4 *B*) raises further questions regarding the role of ubiquitin marking. If this conjugate and a similar size conjugate observed in permeabilized mitotic and interphase rat hepatoma cells (48) are the same, then cell cycle-specific conjugation of ubiquitin to certain proteins may be sensitive to conditions that change after cellular permeabilization. The 26,000 conjugate formed at mitosis in vivo could be a proteolytic intermediate of an 18,000-mol-wt protein degraded only at metaphase, or the conjugate might reflect regulation through ubiquitin marking. Inability to form a similar conjugate in ts85 cells could explain why they arrest in G2 at nonpermissive temperatures (15).

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