

EVIDENCE FOR THE AUTOPHAGY OF MICROINJECTED PROTEINS IN HELA CELLS

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ABSTRACT

Rhodamine-conjugated proteins were microinjected into living HeLa cells. Fluorescence microscopy was then employed to study their segregation from the cytoplasm into lysosomes. Results obtained in this way were verified when the corresponding unconjugated proteins were localized by autoradiographic, histological, and antibody-staining methods after their microinjection. Most injected proteins were segregated into cytoplasmic granular structures during their removal from cells. As evidence that these were autophagic vacuoles, they were found to contain no detectable acid phosphatase activity upon formation, after which they moved to the juxtannuclear position of lysosomes and appeared to fuse with them. The segregation of microinjected proteins exhibited a high degree of selectivity. The half-times of placement of individual exogenous proteins into cytoplasmic granules varied from 3 h to nearly 3 days, and one protein, hemoglobin, was never observed to enter them. Furthermore, endogenous HeLa proteins in a size fraction near 200,000 daltons were segregated much more rapidly than those in a fraction near 40,000 daltons. In these studies, rapid protein segregation appeared to take place by a mechanism of exclusion of the injected protein from numerous cytoplasmic domains.

KEY WORDS microinjection · autophagy · lysosomes · protein degradation

The means by which a cell normally degrades its own cytoplasmic proteins is not well understood. However, it is known that various soluble proteins turn over at vastly different rates within cells (9). Therefore, the mechanism(s) responsible for their degradation must be highly selective. Correlations have been made between turnover rates of individual proteins and their susceptibilities to cytoplasmic proteases (8), denaturation (2, 11), size (6), and charge (7). It is possible, though, that many cellular proteins are degraded within lysosomes (5, 17). Selectivity in lysosomal degradation has been postulated to result from variations in rates of denaturation (4) or hydrolysis (14) of proteins within them. In addition, it is possible that the selective enclosure of proteins within

lysosomes (autophagy) might be responsible for variations in the rates of intracellular protein degradation (13).

In this work, we have attempted to demonstrate that many cytoplasmic proteins are selectively enclosed within autophagic vacuoles as evidence that autophagy is responsible for the selective degradation of many cytoplasmic proteins. For this demonstration, various proteins were conjugated with rhodamine and microinjected into living HeLa cells. Fluorescence microscopy was then employed to determine their intracellular localization during removal from the cell.

MATERIALS AND METHODS

Microinjection and Cell Culture

Microinjection was performed as previously described (15). For each injection experiment, numerous (100-

300) cells on each of several cover slips received cytoplasmic injections. Injected cells on individual cover slips were then photographed under fluorescence optics at various times thereafter. Injections varying from ~2 to 10% (15) of the cell volume were made for each determination without alterations in the observations reported. Phase-contrast observations of the injected cells were routinely made to ensure that they had not been damaged by the injections. Cells with an abnormal appearance under phase-contrast optics were not considered. All injections were made into randomly selected interphase cells.

HeLa cells were the gift of Dr. E. G. Diacumakos, and were cultured and prepared for injection as described (15). Chick embryo fibroblasts (CEF) were prepared as described by Hanafusa (10).

Conjugation of Proteins with the Fluorescent Marker

Myoglobin type 1, β -lactoglobulin, ovalbumin grade V, human hemoglobin grade V, bovine serum albumin (BSA) fraction B, and thyroglobulin type 1 (Sigma Chemical Co., St. Louis, Mo.); catalase, and β -galactosidase (Worthington Biochemical Corp., Freehold, N. J.); and horse heart apoferritin and human immunoglobulin (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) were conjugated with rhodamine as follows: a 4–8 mg/ml protein solution in 0.10 M sodium phosphate, pH 9.0, was mixed with tetramethylrhodamide isothiocyanate (RITC; isomer R, BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) at 30–60 μ g/mg protein and incubated at 4°C overnight. The reaction mixture was then passed over a Bio-Gel P-2 or P-6 (Bio-Rad Laboratories, Richmond, Calif.) filtration column to remove unreacted RITC. The conjugated protein was further purified by a second molecular exclusion chromatography step. For this purpose, the appropriate gel was packed in a 1.5 \times 150-cm column and eluted with 200 ml of distilled water or 0.01 M sodium phosphate, pH 7.2 (6 ml/h) (Bio-Gel P-60 was used for myoglobin, β -lactoglobulin, and ovalbumin; Sephadex G-100 for hemoglobin and BSA; Sephadex G-200 for immunoglobulin; and Bio-Gel A-1.5m for apoferritin, catalase, and thyroglobulin). Conjugated-protein fractions, corresponding in size to the original intact protein molecule, were lyophilized after extensive dialysis, and redissolved in a small volume of 0.15 M KCl solution for injection. The protein concentration and dye-to-protein ratios were determined by the method of Amante et al. (1). After the final purification step, the conjugate was also analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis essentially according to Laemmli (12). In each case, the purified conjugated proteins were resolved into bands characteristic of the original molecule. In addition, under nonreducing conditions the dye migrated with each of the proteins, indicating that it was covalently attached to them.

Endogenous HeLa soluble proteins were prepared from HeLa S-3 cells (a gift of Dr. J. E. Darnell). The cells were washed in phosphate-buffered saline and the plasma membranes disrupted with a VirTis homogenizer (VirTis Company, Gardiner, N. Y.). The homogenate was centrifuged at low speed (Sorvall SS32 rotor at 10,000 rpm for 10 min [Ivan Sorvall, Inc., Norwalk, Conn.]) to remove debris, and at high speed (Beckman SW 39 rotor [Beckman Instruments Inc., Science Essentials Co., Mountainside, N. J.] at 35,000 rpm for 5 h) to remove ribosomes. Proteins in the postribosomal supernate were conjugated with RITC, as described above, and passed over a Bio-Gel A-0.5m gel filtration column previously calibrated with known standards; 40,000-dalton and 200,000-dalton fractions were collected and concentrated as follows.

Protein Concentration

Endogenous HeLa proteins were concentrated using fine (Bio-Fiber 50, Bio-Rad, 1 mm in diameter) dialysis tubing. One open end of a 30-cm segment of the tubing was secured near the bottom of a vessel containing diluted protein solution. The solution was then withdrawn into the tubing to within 1 cm of the free end which was then clamped shut. The entire length of the solution-filled tubing was covered with Aquacide II (Calbiochem, San Diego, Calif.) and placed in a cool, moist chamber until the protein solution within the tubing near the clamped end became sufficiently concentrated, usually within 60 min. The tubing was removed from the Aquacide and immersed in 0.15 M KCl solution for 15 min at 4°C. The concentrated solution was removed and used for injection.

Fluorescence Microscopy

Fluorescence photography was performed upon unfixed cells before phase-contrast or dark-field photography. All photographs were taken at \times 400. Tri-X (Kodak) film was exposed for 1–5 min using #546 and #65 filters (Carl Zeiss, Inc., New York) for excitation and emission, respectively. Antibody staining and photography were performed as previously described (15). The anti-rat-serum-albumin serum was prepared by injecting rat serum albumin (Sigma, fraction B; 5 mg) along with Freund's complete adjuvant (Calbiochem) intramuscularly into rabbits at 1-mo intervals. Antiserum was collected 1 wk after the final injection and used only after it was shown to produce only one precipitin line in Ouchterlony plates against purified rat serum albumin and rat serum. Injected cells were fixed in methanol acetone (3:1).

Autoradiography was performed as described (15) after fluorescence microscopy. 125 I-modified BSA was obtained from New England Nuclear, Boston, Mass.

Acid Phosphatase Stain

Acid phosphatase was localized within HeLa cells according to the naphthol AS-BI phosphate-hexazonium

parosaniline method of Barka and Anderson (3) after fluorescence photography. Photographs of stained cells were taken through a #546 filter to enhance contrast between stained and unstained areas. The benzidine stains were performed upon methanol fixed cells as described (15).

Vinblastine sulfate (Sigma Chemical Co.) and cytochalasin B (Aldrich Chemical Co., Milwaukee, Wis.) were dissolved in growth medium and diluted to 0.01, 0.1, 1.0, and 10 $\mu\text{g}/\text{ml}$. For inhibitor studies, HeLa cells were transferred to these solutions immediately after being injected with the conjugated proteins. For incubation at 4°C, cells were placed in a precooled chamber having an atmosphere of 5% CO₂ in air.

RESULTS

Segregation of BSA

BSA was conjugated with tetramethylrhodamine isothiocyanate. The conjugate was purified by molecular exclusion chromatography and analyzed by polyacrylamide gel electrophoresis to ensure that all rhodamine in the sample was covalently associated with protein molecules of the appropriate molecular weight. The purified conjugate (Rh-BSA) was then microinjected into numerous HeLa cells on each of several cover slips. Fluorescence micrographs were taken of the injected cells immediately and at various times thereafter to determine the intracellular localization of the BSA-associated fluorescence (and presumably BSA itself) during its removal from within the cells. Fig. 1 illustrates that although the fluorescence was uniformly distributed within the HeLa cell cytoplasm immediately after injection (Fig. 1*a*), within 3 h after injection most of it was found in what appeared to be granules which were scattered throughout the cytoplasm (Fig. 1*b*). After 10 h (Fig. 1*c*), the fluorescent granules had moved together near the nucleus. Similar results were obtained after injection of Rh-BSA into chick embryo fibroblasts.

Examination of Fig. 1*c* and *d* shows that the BSA-containing cells appeared entirely normal with respect to neighboring uninjected cells in phase-contrast optics. In addition, fluorescent granules did not correspond to structures readily apparent in either phase-contrast or dark-field optics (Fig. 1*d*, *f*, and *g*). Comparison of the fluorescence within cells immediately after injection to that remaining 36 h thereafter suggested that much of the originally injected fluorescence had been removed from the cells during the 36-h incubation. However, because of the minute amounts of protein injected, it was not possible

to verify that its disappearance was due to its degradation.

Two questions were raised by these observations. First, did the rhodamine remain associated with the BSA molecules throughout the duration of the experiment, and if so, did the attached rhodamine promote placement of the BSA molecules into the granule-like structures? Second, what type of granules (or vacuoles) were involved? When free Rhodamine B dye (1 mg/ml) was injected into the HeLa cells, it diffused out of them within 2 min after its microinjection, indicating that intracellular fluorescence was due to protein-associated rhodamine.

To demonstrate that the Rh-BSA had not been moved into granules as a consequence of the attached rhodamine molecules, a solution of ¹²⁵I-modified BSA (¹²⁵I-BSA) was combined with a solution of Rh-BSA, and the mixture was injected into HeLa cells. At various times thereafter, the intracellular distributions of fluorescence and radioactivity were compared. Immediately after injection, both fluorescence and radioactivity were evenly distributed throughout the cytoplasm of injected cells. However, after 10 h, both labels had been moved to identical juxtannuclear positions within the cells (Fig. 2). Thus, ¹²⁵I-BSA had been segregated from the cytoplasm exactly as Rh-BSA had been. Furthermore, when unlabeled rat serum albumin was microinjected into HeLa cells, it too was moved into a juxtannuclear position within 10 h after injection, as revealed by staining with a specific antibody. Therefore, movement of Rh-BSA into juxtannuclear granules appeared to be due to the characteristics of the BSA molecule and not to the attached rhodamine molecule or even to the presence of rhodamine within the injected cell. Furthermore, rhodamine conjugation was shown to provide a reliable means of localizing injected BSA, and should be useful as an intracellular tracer of other protein molecules.

It is apparent from the autoradiographs prepared after injections that little of the originally injected ¹²⁵I-BSA had escaped movement into a juxtannuclear position 10 h after injection. This indicated that the injected protein had been efficiently segregated from bulk cellular proteins and highly concentrated into the granule-like structures.

Identification of Autophagic Vacuoles

Autophagic vacuoles have been postulated to be the initial sites of cellular protein segregation

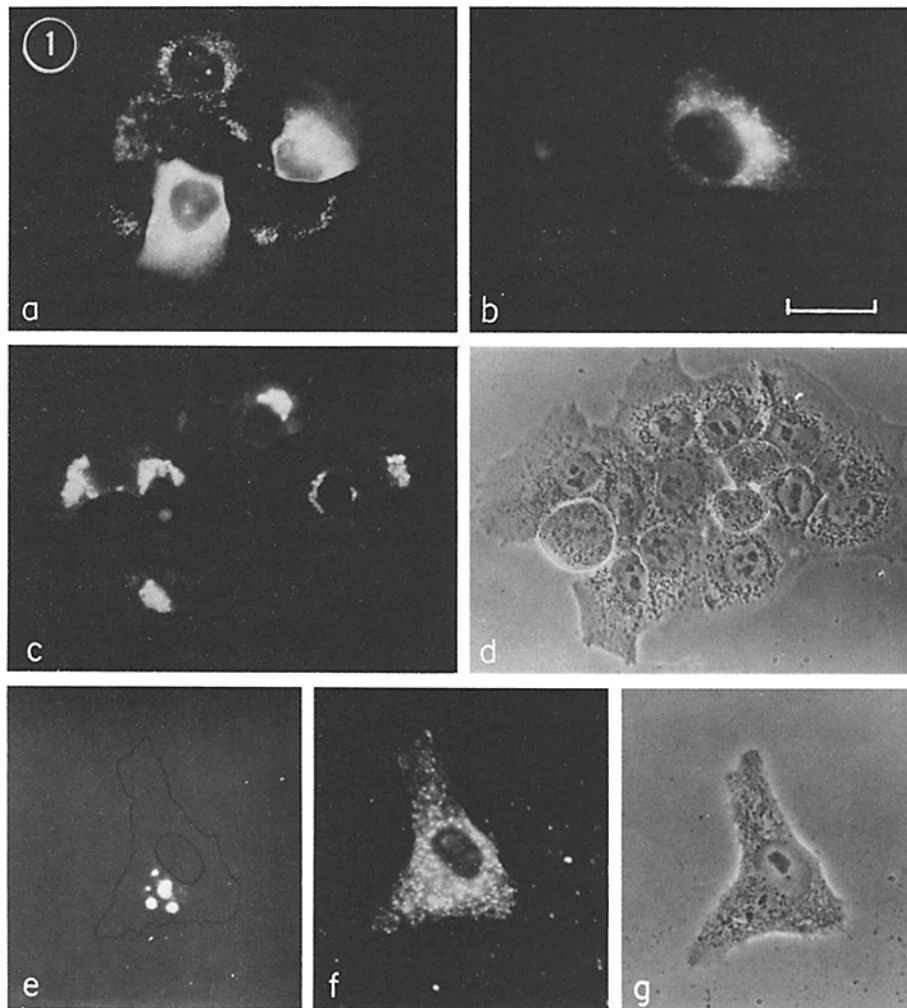


FIGURE 1 Segregation of microinjected rhodamine-conjugated bovine serum albumin into cytoplasmic vacuoles. Fluorescence micrographs were taken (a) immediately, (b) 3 h, (c) 10 h, and (e) 36 h after microinjections of Rh-BSA (15 mg/ml) into HeLa cells. 3 h after injection, fluorescence is found within granular structures throughout the cytoplasm (b) which then accumulate near the nucleus (c). Duplicate phase-contrast (d, g) and dark-field (f) photographs are presented. Similar results were obtained with Rh-BSA concentrations from 10 to 20 mg/ml. Some background light-scatter is detectable from vacuoles around the nuclei of injected cells, particularly in frame (a). Bar, 20 μm (13 μm in frames c and d).

during autophagy. If the granule-like structures into which Rh-BSA was segregated were autophagic vacuoles, they should be identified as such immediately after their formation by their content of fluorescent protein segregated from the cytoplasm, and by their lack of lysosomal-specific acid phosphatase (AP) activity. After formation, autophagic vacuoles would be expected to fuse with lysosomes. Both of these criteria are considered

in the experiments illustrated in Fig. 3. Rh-BSA was injected into HeLa cells. At various times thereafter the Rh-BSA distribution within the cells was determined by fluorescence microscopy and compared to the distribution of AP activity as determined immediately thereafter by the method of Barka and Anderson (3).

Immediately after injection (Fig. 3a), the BSA-associated fluorescence was evenly distributed

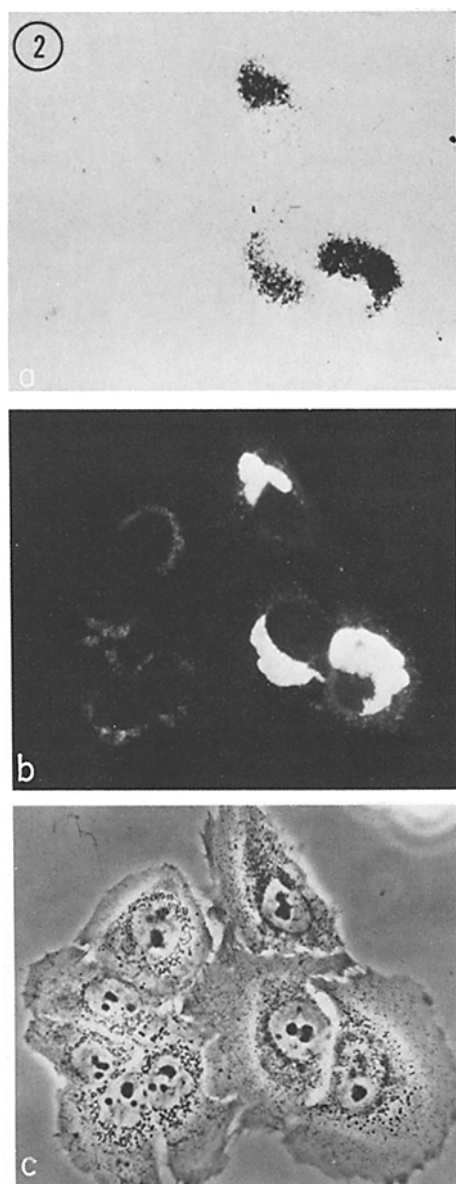


FIGURE 2 Cosegregation of Rh-BSA and ^{125}I -BSA. A solution of rhodamine-conjugated BSA (10 mg/ml) was combined with an equally concentrated solution of ^{125}I -BSA and the combined solution injected into HeLa cells. 10 h later these fluorescence (b) and phase-contrast (c) photographs were taken and the cells were fixed immediately for autoradiography (a). The cells were positioned identically within each frame. The fluorescent and radiolabeled BSA were segregated into identical positions within these cells (within the limits of autoradiographic resolution).

throughout the cytoplasm and the AP activity was localized in the usual juxtanuclear granules (lysosomes). 3 h after injection (Fig. 3b), fluorescence was found in granules throughout the cytoplasm which contained no detectable AP activity. This activity was still localized in apparently undisturbed juxtanuclear lysosomes. However, more than 7 h after injection, the fluorescence and AP activity became coincident within a juxtanuclear position of the cell (Fig. 3c), suggesting that the fluorescent granules had moved to and fused with the lysosomes. AP activity thereafter occupied a much greater than normal area within the cells, and was at first less distinct, suggesting that the lysosomal enzymes had been diluted by the formation of a large number of secondary lysosomes after fusion with autophagic vacuoles. During the remainder of the experiment, the AP activity of the cell increased in intensity and the fluorescence gradually disappeared (Fig. 3d-f).

Although these observations provide no direct proof that the granules actually fused with lysosomes (thus identifying them as autophagic vacuoles), the observed association of fluorescence and AP activity strongly suggests that fusion did take place. Hereafter the fluorescent, granule-like structures formed after the microinjection of conjugated proteins will be termed vacuoles, even though it has not been proven that they are membrane-bounded. It is interesting to note the similar distributions of both fluorescence and AP activity in Fig. 3c. All the fluorescent vacuoles appeared to have fused with lysosomes within 4 h after their formation. The result was an even distribution of both enzyme and segregated protein throughout the lysosomal system of the cells. Furthermore, few, if any, lysosomes appeared to have escaped fusion with fluorescent vacuoles, as no areas remained within the injected cells which exhibited AP activities comparable to that of unaltered lysosomes. In addition, it appeared from Fig. 3e and f that after the fusion of large numbers of vacuoles with lysosomes, the cell responded by greatly increasing its total content of AP activity.

The dynamics of formation of these vacuoles and their apparent fusion with lysosomes was further analyzed by the use of inhibitors. Neither the microtubule inhibitor, vinblastine sulfate, nor the microfilament inhibitor, cytochalasin B, blocked either process at concentrations from 0.01 to 10 $\mu\text{g}/\text{ml}$, even though definite cytopathic

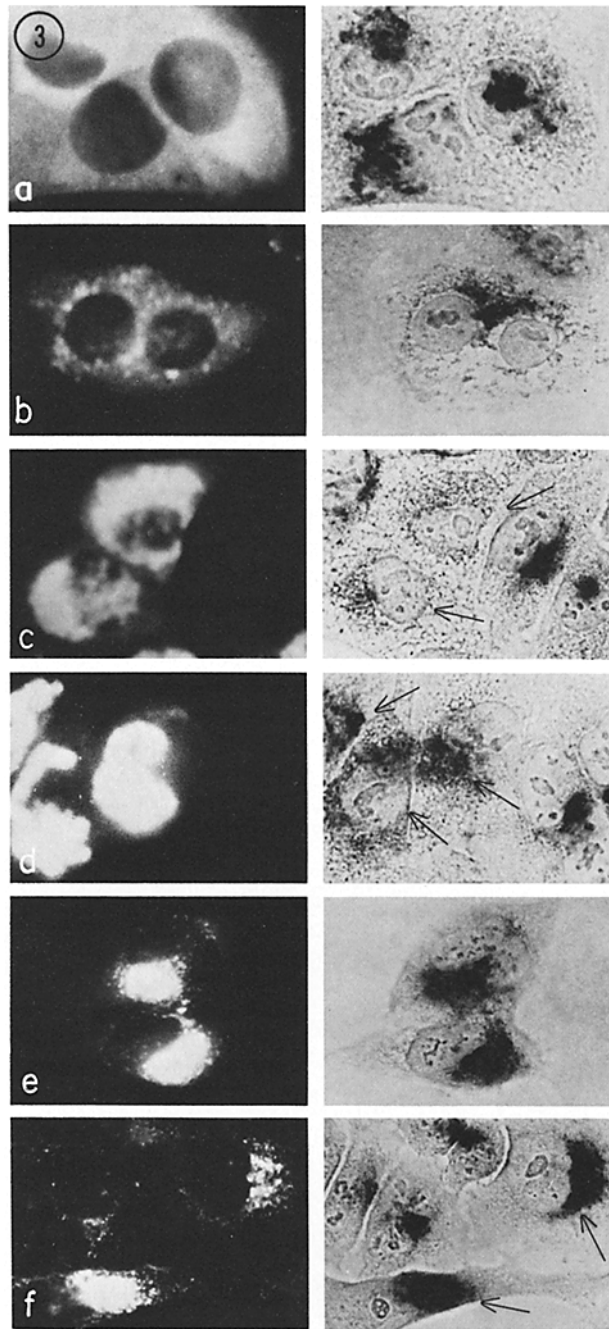


FIGURE 3 Identification of autophagic vacuoles. Rh-BSA (15 mg/ml) was microinjected into numerous cells on each of 6 cover slips. Immediately (*a*), and (*b*) at 3 h, (*c*) 7 h, (*d*) 10 h, (*e*) 25 h, and (*f*) 50 h thereafter these fluorescence (left) photographs were taken, and the cells were stained immediately for acid phosphatase activity (duplicate photographs at right). Arrows indicate injected cells unless all cells within a photograph received injections. The normal distribution and intensity of acid phosphatase within HeLa cells is shown in uninjected cells. More than 50 h (*f*) after injections, few cells still contained identifiable fluorescence. Those which did contain fluorescence and others which could be identified as having received injections, contained large amounts of acid phosphatase.

effects were observed in cells cultured at the higher inhibitor concentrations. However, incubation of the cells at 4°C immediately after injection of Rh-BSA completely blocked its movement into vacuoles. This effect was reversible (by restoring the cells to 37°C) after up to 6 h incubation in the cold. After longer exposures the cells appeared to lose viability.

Apparent Autophagy of Other Exogenous Proteins

Various other proteins were conjugated, purified, and microinjected into HeLa cells, and their intracellular distributions were analyzed as BSA had been. The results of these analyses are summarized in Table I. With the exception of hemoglobin, and perhaps immunoglobulin, all exogenous proteins had moved into vacuoles which were presumed to be autophagic vacuoles, because they were formed throughout the cytoplasm and then moved to a juxtannuclear position exactly as in the case of the BSA. As evidence that this segregation was highly selective, its rate for individual proteins varied greatly; half of the protein-associated fluorescence appeared in vacuoles in <3 h with BSA and thyroglobulin, but a corresponding segregation of ovalbumin took nearly 3 days. Within this limited set of exogenous proteins, there was little correlation between more rapid rates of segregation and high molecular

weight of the individual proteins (6), their acidic pI (7), or high rhodamine to protein ratios; and there was no apparent correlation between rates of segregation and intracellular concentrations of the microinjected protein.

The results with hemoglobin were interesting because neither conjugated human nor duck hemoglobins were ever observed to enter vacuoles in either HeLa cells or CEF. Strikingly similar results were obtained when unlabeled duck hemoglobin was localized within HeLa cells using specific antibodies (15). In addition, when unmodified human hemoglobin was localized after injection into HeLa cells with the benzidine stain, the hemoglobin-associated peroxidase activity was observed to remain totally randomly distributed throughout the cell and free from vacuolization, while it gradually disappeared. Thus, results obtained with three independent techniques of hemoglobin localization confirm that it was not moved into vacuoles by HeLa cells, and that conjugation and fluorescence microscopy provided a reliable method for monitoring the intracellular behavior of hemoglobin (as was found for BSA). Finally, it is apparent from these results that conjugation with rhodamine was not sufficient in itself to promote the movement of an injected protein into autophagic vacuoles.

As a further example of the selectivity of the vacuolization of injected proteins by cells, it was found that when anti-duck hemoglobin immuno-

TABLE I
Segregation of Exogenous Rhodamine-Conjugated Proteins into Vacuoles after Microinjection into HeLa Cells

	Entry into vacuoles	Mol. wt. × 10 ⁻³ daltons	Protein subunits	pI*	Protein Conc. mg/ml	Dye to protein ratio‡
Myoglobin	+	13	1	6.9	21	ND§
β-lactoglobulin	++++	37	2	5.2	65	0.14
Ovalbumin	+	45	1	4.6	40	0.09
Hemoglobin	-	68	4	7.1	60	ND
BSA	++++	66	1	4.8	15	0.24
Immunoglobulin	+,-	160	4	7.5	25	0.12
Catalase	++	232	4	5.4	30	0.28
Apo ferritin	++	480	24	4.4	20	0.51
β-galactosidase	+++	540	4		28	0.39
Thyroglobulin	++++	669	2	4.6	60	0.30

Purified, rhodamine-conjugated exogenous proteins were microinjected into HeLa cells. The placement of protein-associated fluorescence in vacuoles was monitored thereafter by fluorescence microscopy. Rates of entry into vacuoles varied from under 3 h (++++) to nearly 3 days (+) for one-half of the fluorescence to be found in vacuoles. Immunoglobulin entered vacuoles of CEF (after 24 h following injection), but not those of HeLa cells.

* pI of the unconjugated protein.

‡ Rhodamine molecules per 1 × 10⁴ daltons of protein.

§ ND, not determined.

globulin was injected into HeLa cells, either just before or just after a separate injection of rhodamine-conjugated duck hemoglobin. duck-hemoglobin-associated fluorescence was found in vacuoles within 2 h of the second injection. If sufficient immunoglobulin was injected, all fluorescent hemoglobin was found in vacuoles. Smaller immunoglobulin injections promoted the segregation of only a part of the intracellular antigen. This observation suggests that antigen-antibody equivalence (and perhaps intracellular precipitate formation) was not required for this rapid segregation. If rapid segregation were the characteristic of all intracellular antigen-antibody complexes, it might be possible to selectively remove any normal antigen from within the living cell by microinjection of the appropriate antibody.

Placement of Endogenous Proteins into Vacuoles

As a final evidence that segregation of injected proteins was highly selective within HeLa cells, its rate for endogenous proteins was found to be strongly influenced by molecular weight. Total HeLa S-3 cytoplasmic proteins were conjugated with rhodamine, separated by molecular exclusion chromatography into a 40,000- and a 200,000-dalton fraction, and each fraction was separately microinjected into HeLa cells. By visual estimate, four-fifths of the 200,000-dalton fraction, but only one-fifth of the 40,000-dalton fraction was found in vacuoles 33 h after injection. After 55 h, essentially all the fluorescence of the larger protein fraction, but only approximately half that of the smaller fraction was found in vacuoles. These results are interesting in relation to those of Dice et al. (6) who found that in a variety of mammalian tissues, as well as in HeLa cells, larger proteins turn over more rapidly than do smaller ones. It is important to note that almost all the fluorescence associated with the endogenous proteins eventually entered vacuoles with characteristics similar to those formed after the injection of Rh-BSA, suggesting that many HeLa cellular proteins eventually enter lysosomes as a normal event in their breakdown.

The Mechanism of Segregation

In an attempt to more closely analyze the mechanism by which proteins free in the cytoplasm became enclosed within vacuoles, highly

concentrated solutions (25–40 mg/ml) of Rh-BSA or Rh-thyroglobulin were injected into HeLa cells, and fluorescence photographs were frequently taken in the next several hours. As can be seen in Fig. 4, fluorescent protein became evenly distributed throughout the cytoplasm immediately after the injections. But within a period as short as 30 min (Fig. 4*b*), areas appeared within the cytoplasm from which fluorescent protein had been excluded. These areas grew in size (up to ~1–2 μm) and in number, until at 3 h after injection (Fig. 4*d*) the entire cytoplasmic area had become filled with these excluded areas, and the fluorescence was found in a reticulum-like arrangement at their boundaries (Fig. 4*e*). This fluorescence then condensed down upon the nucleus (Fig. 4*f*) and became coincident with AP activity (photograph not shown). Although this sequence of segregation by exclusion may take place only after injections of highly concentrated Rh-BSA or Rh-thyroglobulin into HeLa cells, it is possible that the sequence represents the normal mechanism of autophagic segregation, made apparent because it was forced to operate so extensively. Therefore, the reticulum-like arrangement of segregated protein observed might simply represent the microscopic appearance of a large number of vacuoles.

Another interesting observation was frequently made after the injection of highly concentrated Rh-BSA or Rh-thyroglobulin solutions into HeLa cells. After movement of the segregated protein into a juxtannuclear position, instead of remaining in this position and gradually disappearing from the cell as observed with less concentrated solutions as previously described, the entire segregated protein mass was pushed up out of the cell and released in its entirety by the cell in a manner reminiscent of the extrusion of the mammalian erythrocyte nucleus.

DISCUSSION

The technique of microinjection of rhodamine-conjugated proteins was employed to facilitate the direct observation of many proteins during their degradation within living HeLa cells. It was found that most injected proteins were segregated from the cytoplasm into granule-like structures before their disappearance from within the cells. As evidence that these granules were autophagic vacuoles, it appeared (in the case of BSA) that they fused with lysosomes. The evi-

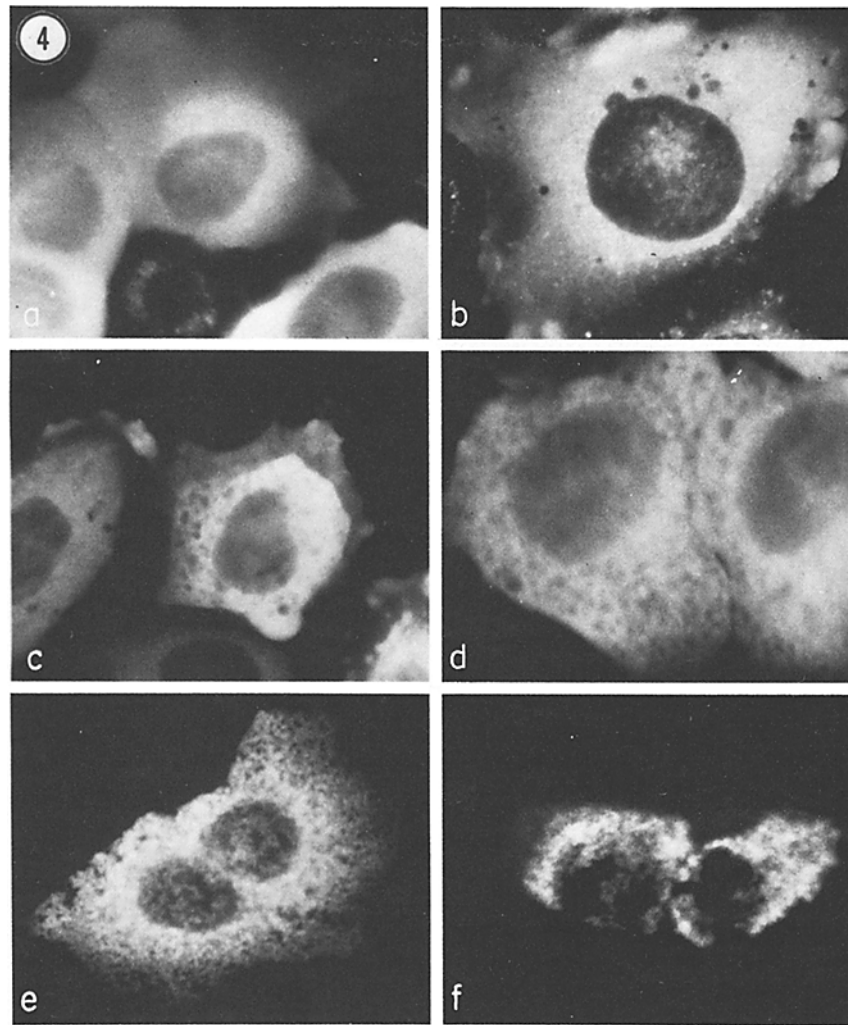


FIGURE 4 Observations on the mechanism of rapid protein segregation. Highly concentrated (24–40 mg/ml) Rh-BSA was microinjected into HeLa cells on several cover slips. These fluorescence photographs were taken of injected cells (a) immediately, (b) 30 min, (c) 1 h, (d) 1½ h, (e) 3 h, and (f) 6 h thereafter. Areas appeared within the cytoplasm of injected cells from which fluorescent protein had been removed starting within 30 min after injections (b). These areas grew in size and number (c) until the entire cytoplasmic area of the cell became involved (d) and the fluorescent protein finally became concentrated into a reticulum-like arrangement (e) which then condensed down upon the nucleus (f) and fused with lysosomes. This figure represents the most rapid segregation observed in these types of experiments. Analogous results were obtained with rhodamine-conjugated thyroglobulin.

dence for this conclusion is as follows: after their formation throughout the cytoplasm, the fluorescent granules moved to the juxtannuclear position of lysosomes, after which the fluorescence and acid phosphatase activity appeared to occupy the same position within the cell. In this process, the distribution and intensity of the AP activity was greatly altered so that it appeared to occupy

a greater cellular area and to be less intense than normal. These observations are consistent with the explanation that upon fusion with the large number of the Rh-BSA-containing granules, the AP activity was diluted into a large number of secondary lysosomes. Thereafter, the fluorescence gradually disappeared from the cell as the intensity of the AP activity greatly in-

creased while continuing to occupy a larger cellular area than normal. This apparent fusion of the granules with lysosomes, along with their vacuolar appearance and the fact that they contained protein segregated from the cytoplasm, constitute the evidence that they were autophagic vacuoles and that the injected proteins were degraded by autophagy.

Further evidence for this assertion comes from the observed selectivity of the segregation of microinjected proteins. The rates of segregation for individual exogenous proteins varied greatly from 3 h to nearly 3 days. In addition, large endogenous HeLa proteins moved into vacuoles more rapidly than smaller ones. These observations correlated well with numerous chemical studies which indicate that protein turnover is highly selective (9), and that larger proteins turn over more rapidly than smaller ones (6). Notwithstanding the fact that only microinjected proteins have been analyzed in this work, these correlations suggest that the apparent autophagic segregation described in this work may be related to protein turnover in general. They also raise the possibility that the selectivity of autophagy may play an important role in determining the rates of degradation of many cellular proteins. However, these observations must be interpreted with caution until direct evidence is obtained to demonstrate that (a) the disappearance of fluorescence from injected cells corresponds to hydrolysis of conjugated proteins, and (b) the fluorescent, granular structures are actually membrane-bound.

Most of the proteins injected into HeLa cells were eventually found in granules similar to those containing Rh-BSA as previously described. This was the case whether the proteins were exogenous or endogenous, conjugated or unconjugated. However, it should be emphasized that hemoglobin was never observed to enter granular structures during its disappearance from the cell, indicating that autophagy is not the only mechanism for cellular protein degradation.

Most of these conclusions were based upon observations of the intracellular distributions of fluorescence after the microinjection of rhodamine-conjugated proteins. Such observations were shown to provide a reliable indication of the intracellular behaviors of the injected proteins themselves. For example, when unconjugated hemoglobin or serum albumins were mi-

croinjected into HeLa cells, and the proteins subsequently localized within the cells using specific antibodies, autoradiography, or histological staining techniques, the results exactly duplicated those obtained with rhodamine conjugation and fluorescence microscopy. Furthermore, it is not likely that the injected cells described in this work display unusual behavior. Microinjected cells have been carefully analyzed, and were found to carry on cell division, enzyme induction, globin mRNA translation (15) and virus production (16) normally after injections of a variety of materials. Furthermore, such studies indicated that injected macromolecules behaved within the injected cells as they would have behaved within the cells from which they were isolated.

When a large amount of rapidly segregated protein was injected into HeLa cells, segregation of injected protein appeared to take place by a process of exclusion from many small domains throughout the cytoplasm. Although this process may not be characteristic of normal autophagy, it is interesting that the cell possesses the capacity to carry it out. Observations of the fine structure of the cell around these areas of protein exclusion might provide valuable information concerning the subcellular elements involved in autophagic segregation, and perhaps suggest how they function.

This work also establishes the value of microinjection in further studies of the biochemistry of autophagic segregation. Various proteins were segregated at greatly different rates, indicating that some distinguishing molecular characteristics had been recognized by the autophagic segregation system of the HeLa cell. As one means of directly determining what these molecular characteristics might be, various chemical or biological modifications could be introduced into the test proteins. The effects of these modifications upon the rate of segregation of the modified protein could then be determined using microinjection and appropriate tracer techniques.

We thank Dr. Brian Poole for his helpful criticism of this manuscript and Randie Davidson for her help in its preparation.

This work was supported by a National Institutes of Health grant (GM 17383), The American Cancer Society Grant (NP-228G), and the Rockefeller Foundation Program in Reproductive Biology.

Received for publication 24 February 1977, and in revised form 25 July 1977.

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