Degradation and Regurgitation of Extracellular Proteins by Cultured Mouse Peritoneal Macrophages and Baby Hamster Kidney Fibroblasts

KINETIC EVIDENCE THAT THE TRANSFER OF PROTEINS TO LYOSOMES IS NOT IRREVERSIBLE*

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The uptake and degradation of bovine serum albumin (BSA), bovine liver catalase, and rabbit muscle enolase have been studied in cultured mouse peritoneal macrophages (MPM) and baby hamster kidney fibroblasts (BHK cells). Rates constant for the uptake of the three proteins by MPM were similar. In addition, BSA accumulation was independent of BSA concentration in the uptake medium and was not inhibited by a large excess of serum, suggesting that protein accumulation was by fluid phase pinocytosis. Following an overnight uptake, 20–50% of the accumulated protein was subsequently regurgitated into the medium in a trichloroacetic acid/phosphotungstic acid-preparable form. This material co-migrated with the authentic protein during molecular sieve chromatography on Sephadex G-50. The rates of appearance of trichloroacetic acid/phosphotungstic acid-insoluble products were greater than expected for cell death and leakage. The observed first order rate constants, \( k_{obs} \), for the appearance of trichloroacetic acid/phosphotungstic acid-soluble and trichloroacetic acid/phosphotungstic acid-insoluble products in the culture medium were identical, indicating that both products were released in parallel from MPM and BHK cells. The \( k_{obs} \) for intracellular BSA degradation and regurgitation were independent of the initial BSA concentration in the uptake medium, but were decreased about 35% when degradation was allowed to proceed in the presence of high concentrations of serum. Degradation was also inhibited by chloroquine and pepstatin. Inhibition of degradation was accompanied by an increase in the total amount of regurgitated protein appearing in the medium. Remarkably, however, these inhibitors also decreased \( k_{obs} \) for regurgitation, thereby preserving the similarity in the observed rate constants for the appearance of trichloroacetic acid/phosphotungstic acid-soluble and trichloroacetic acid/phosphotungstic acid-insoluble products. These and other results were inconsistent with desorption of proteins from the surface of the culture dish or the surface of cells as the source of the trichloroacetic acid/phosphotungstic acid-insoluble label appearing in the medium. The simplest explanation of our observations is that the trichloroacetic acid/phosphotungstic acid-soluble products and regurgitated proteins arose from a single intracellular pool of labeled substrate whose decay was independent of which product was used to monitor changes in the size of the pool. As this substrate pool appears to correspond to a degradative compartment, we suggest that the transfer of extracellular proteins to lysosomes may not be unidirectional and that protein substrates within the lysosome could reappear in the medium during a subsequent chase.

Most cells appear to have the capacity to internalize soluble extracellular proteins by either fluid phase or adsorptive pinocytosis (1). This phenomenon may be important in metabolic regulation by either initiating or terminating the action of polypeptide hormones (2); in host defense through antigen processing (3) and removal of immune complexes (4), secreted lysosomal hydrolases (5), and \( \alpha \)-macroglobulin-proteinase complexes (6); in molecular transport and delivery of cholesterol (7); and in the removal of altered proteins with recycling of their chemical constituents (8). In most cases, the terminal events are fusion of pinocytic vesicles with lysosomes and subsequent degradation of their resident proteins (1–10). The major products observed with both cultured cells and isolated lysosomes appear to be free amino acids (9, 10). There is also evidence that lysosomes play an important role in the turnover of intracellular proteins (11–15). However, the magnitude of their contribution is still in dispute (for example, compare Refs. 11–15 with 16–20). A number of interesting relationships have been reported between the rates of turnover of cellular proteins in vivo and their size, charge, conformational stability, and susceptibility toward proteolysis in vitro. Based on some of these findings, it has been suggested that the rate-determining step in the degradation of intracellular proteins may be either their denaturation (21) or proteolysis (22) within the lysosome. Since pinocytosis provides a route by which specific labeled proteins can be delivered to lysosomes, its study may provide useful information relevant to the mechanism of intracellular protein turnover. Consequently, one purpose of this work was to develop methods to quantify and kinetically characterize lysosomal protein degradation in cultured cells.

During the course of these studies, it was observed that a significant fraction of labeled substrates which had accumulated in cells by pinocytosis could reappear in the medium as trichloroacetic acid/phosphotungstic acid-preparable radioactivity during a subsequent chase. Cultured cells had previously been observed to regurgitate nondegradable substrates (23, 24) by a process which has been termed retroendocytosis (24). In addition, \(^{125}\)I-labeled low density lipoproteins (25), transferrin (26), and asialoglycoproteins (27), which are internalized by receptor-mediated pathways, can also undergo exocytosis, possibly as a consequence of incomplete dissociation from their receptors following internalization. However, it has been reported that proteins internalized by fluid phase...
pinocytosis do not reappear in the medium in significant amounts during a subsequent chase (28). Furthermore, it has been suggested that if regeneration does occur, it is most likely due to the recycling of endocytic vesicles prior to fusion with lysosomes (29, 30). However, the possibility of substrate cycling from the lysosome has not been rigorously excluded.

In addition, the calculation of degradation rate constants for endocytosed proteins requires the knowledge of whether the endosome or the lysosome is the source of the regenerated material. Consequently, a second purpose of this work was to determine whether proteins delivered to lysosomes could also undergo exocytosis.

EXPERIMENTAL PROCEDURES

Preparation of Labeled Substrates—Bovine liver catalase and rabbit muscle enolase (Sigma) were analyzed by electrophoresis prior to labeling. They were then iodinated using sodium [125I]iodide (Amersham/Searle) and the Bio-Rad Enzymohept kit (Bio-Rad) according to the instructions of the manufacturer. Unreacted label was subsequently removed by exhaustive dialysis. The specific activities of the final products were in the range of 1-2 mCi/mg for both proteins. [125I]-Labeled BSA (1-5 mCi/mg) was a product of Du Pont-New England Nuclear. Some samples were further purified by chromatography on Affi-Gel Blue (31). 14C-Labeled dextran sulfate was prepared from cyogcn bromide-activated dextran sulfate (Type 2000, Pharmacia P-L Biochemicals) and (U-[14C]glucose (Amersham/Searle) and purified by gel filtration (32). Labeled substrates were concentrated by lyophilization, dissolved in media, and filtered through 0.22-µm disposable sterile filters (Millipore).

Cell Culture—Stimulated mouse peritoneal macrophages were elicited by intraperitoneal injection of 0.75 ml of 4% sterile thioglycolate medium into 8- to 12-week-old male Swiss mice (Scientific Small Co.) (33). Cells were collected 3 days later by flushing the peritoneum with Earle's balanced salts solution. The washings were combined in sterile centrifuge tubes and the cells collected by low speed centrifugation. Pooled cells from three to eight mice were resuspended in Earle's balanced salts solution and aliquots counted with a hemocytometer. Aliquots containing 4-9 x 10^6 cells were then distributed to 35 x 10-mm polystyrene culture dishes or to eight 100-mm polystyrene culture dishes (Lamb) containing medium 199 supplemented with 20% fetal calf serum, mycoplasma, and bovine adventitious agents negative, penicillin (100 units/ml), streptomycin sulfate (2.0 µg/ml), and glutamine (2.0 mM) (GIBCO). After 1-3 h, nonadherent cells were removed by aspiration and fresh media added. Incubation was then continued by repeating that under these conditions greater than 90% of the adherent cells are macrophages (33). Cells were maintained at 37°C in an atmosphere of 5% CO_2 in a Forma Scientific incubator equipped with an automatic CO_2 controller. Fibroblasts, low passage baby hamster kidney cells (BHK-Pl/clone 13), were a gift from Dr. Eugene O. Major (Infectious Diseases Branch, National Institute of Health). In addition, the calculation of degradation rate constants for endocytosed proteins requires the knowledge of whether the endosome or the lysosome is the source of the regenerated material. Consequently, a second purpose of this work was to determine whether proteins delivered to lysosomes could also undergo exocytosis.

RESULTS

Analysis of Data—Apparent rate constants (k_app) were calculated independently from progress curves for the accumulation of trichloroacetic acid/phosphotungstic acid-soluble and trichloroacetic acid/phosphotungstic acid-insoluble radioactivity in the medium with time after uptake, using the integrated forms of the respective first order rate equations. These reactions were usually followed for at least three half-lives. This necessitated estimating the end points for the two products, [TCA]_sol, and [TCA]_insol, where TCA represents trichloroacetic acid. For trichloroacetic acid/phosphotungstic acid-soluble products this was accomplished in the following manner. The radioactivity remaining in the cellular monolayer at the end of the experiment was multiplied by R, the fraction of the total label released by the cells during the preceding period which appeared in a trichloroacetic acid/phosphotungstic acid-soluble form. This product was then added to the total trichloroacetic acid/phosphotungstic acid-soluble radioactivity released during the period of observation. Within the limits of experimental error, R was found to be independent of time throughout the period of observation, thereby justifying this calculation. The end point for the appearance of trichloroacetic acid/phosphotungstic acid-insoluble radioactivity was calculated in a similar manner.

The abbreviations used are: BSA, bovine serum albumin; BHK, baby hamster kidney; SDS, sodium dodecyl sulfate; MPF, mouse peritoneal macrophages.
Two models were considered as possible descriptions of the degradation and regurgitation of labeled proteins observed in these studies. Model 1 (Fig. 1) assumed that trichloroacetic acid/phosphotungstic acid-soluble and -insoluble products originated from a common intracellular pool of labeled substrate, presumably the lysosome. Equations 1 and 2 describe the amounts of radioactive remaining in the cell (38).

\[ [(TCA)\text{sol}] = R[P_0(1 - e^{-k_\text{kd}})] \]
\[ [(TCA)\text{ins}] = (1 - R)[P_0(1 - e^{-k_\text{kd}})] \]
\[ \text{[Cell]} = P_0[1 - e^{-k_\text{kd}}] \]

In these equations, \( P_0 \) is the initial amount of labeled protein present in the cell at the start of the chase period, \( k_d \) and \( k_d \) are the intrinsic regurgitation and degradation rate constants, and \( R \) and \( 1 - R \) are, respectively, \( k_d/(k_d + k_r) \) and \( k_d/(k_d + k_r) \). Thus, \( R \) is the fraction of the total rate due to degradation. In addition, \( R \) is the ratio of trichloroacetic acid/phosphotungstic acid-insoluble radioactivity to total products released at each time point (38). This model predicts that the appearance of trichloroacetic acid-insoluble radioactivity should parallel trichloroacetic acid-soluble radioactivity throughout the entire reaction. In addition, \( R \) and \( 1 - R \) correspond to the total amount of trichloroacetic acid/phosphotungstic acid-soluble and total insoluble radioactive released into the medium were the reaction to proceed to completion, \( [(TCA)\text{sol}] \) and \( [(TCA)\text{ins}] \), respectively. Therefore, Equations 1 and 2 predict that plots of \( \ln(1 - [(TCA)\text{sol}]/[(TCA)\text{ins}]) \) and \( \ln(1 - [(TCA)\text{sol}]/[(TCA)\text{ins}]) \) should yield straight lines with identical slopes corresponding to \( k_{\text{obs}} = (k_d + k_r) \). The intrinsic degradation and regurgitation rate constants can then be calculated from the relationships, \( k_d = Rk_{\text{obs}} \) and \( k_r = (1 - R)k_{\text{obs}} \).

Model 2 assumes that trichloroacetic acid/phosphotungstic acid-soluble and -insoluble radioactivity each arise from a different intracellular compartment, possibly corresponding to the lysosome and the endosome, respectively. Furthermore, transfer of proteins from the endosome to the lysosome is presumed to occur during the chase period. This model is illustrated in Fig. 1. The integrated forms of the rate equations have been derived for this model in the Appendix (Equations 7 and 13) and are presented below.

\[ [(TCA)\text{sol}] = [(TCA)\text{ins}] = P_0[1 - e^{-k_\text{kd}}] + ABP_0[1 - e^{-k_\text{kd}}] \]
\[ A = k_d/(k_d - (k_h + k_l)); \quad B = k_d/(k_d + k_l); \quad C = k_d/(k_d + k_l) \]

In Equations 4–6, and Fig. 1, \( P_0 \) and \( P_0 \) correspond to the amount of protein initially present in the lysosome and the endosome at the beginning of the chase period. The constants, \( k_d \) and \( k_h \) correspond, respectively, to the intrinsic rate constants for exocytosis from the endosome, transfer of proteins from the endosome to the lysosome, and degradation in the lysosome. Furthermore, the sum of the rate constants \( (k_d + k_h) \) determines the observed rate constant and thus the half-life of protein substrates in the endosome. The term \( C_{\text{PEO}} \) corresponds to the end point \( [(TCA)\text{ins}] \). The half-life of the endosome has been estimated to be about 5–10 min for macrophages and fibroblasts (24, 39). The half-lives for the degradation of various extracellular proteins in the lysosome have been reported to be much greater (9, 28, 34) so that \( (k_d + k_h) \gg k_d \). Therefore, model 2 and Equations 4–6 (unlike model 1) predict that trichloroacetic acid-soluble products should continue to appear in the medium well after the regurgitation of trichloroacetic acid-insoluble products has ceased. The condition \( (k_d + k_h) \gg k_d \) also permits a considerable simplification of Equation 4 since the term \( ABP_0 \) becomes insignificant and the term \( [(P_0 - AP_0)/[(TCA)\text{sol}] \) becomes equal to the end point, \( [(TCA)\text{ins}] \). Consequently, under these conditions endosomes should make little contribution to the appearance of trichloroacetic acid/phosphotungstic acid-soluble radioactivity. Accordingly, plots of \( \ln(1 - [(TCA)\text{sol}]/[(TCA)\text{ins}]) \) and \( \ln(1 - [(TCA)\text{sol}]/[(TCA)\text{ins}]) \) against time should yield two different observed rate constants corresponding to \( k_d \) and \( (k_d + k_h) \), respectively. These conclusions have been directly confirmed by simulated calculations based on Equations 4–6. Furthermore, equations similar to these have also been derived for the cases in which the trichloroacetic acid/phosphotungstic acid-precipitable radioactivity arises from desorption of labeled protein bound either to the surface of cells or to the surface of the culture dish. In all of these cases in which a different pool of labeled substrate is assumed to be the source of each of the two products, different observed rate constants are expected to characterize the appearance of the two products.

Accumulation of Labeled Substrates by Cultured Cells—Fig. 2 presents the time course for the accumulation of the nondigestible substrate, 14C-labeled dextran sulfate, when present in the culture medium at an initial concentration of 5 mg/ml. In order to compare the accumulation of different substrates by different preparations of cells, we have normalized intracellular radioactivity by dividing by the specific radioactivities and concentrations of the substrate in the uptake medium and by the number of cells. The quotient has the units of micromolars/10^6 cells plated. It can be seen that the accumulation of dextran sulfate appears linear for about 20 h and then begins to level off by 60 h. From the linear portion of the curve, the initial rate of accumulation was calculated to be about 0.34 µl/h/10^6 cells plated. Fig. 2 also gives the progress curve for the accumulation of BSA (4.5 µg/ml, 1 mCi/mg). In this case accumulation was only linear for about 5 h, suggesting that the approach to the plateau reflected a
As shown in Table I, the accumulation of $^{125}$I-labeled BSA in the absence of serum, when expressed in units of micro Ci/mg labeled protein, each time point represents a separate 35 × 10-mm culture dish plated with 3 × 10$^6$ cells. $^{b}$, endocytosis of $^{131}$I-Iodo-BSA by cultured macrophages. Cells were cultured as described in a in the presence of 4.5 μCi/ml labeled protein. Each incubation was performed in 2.5 × 10$^5$ cells and incubated in the absence (O) and presence (E) of 1 mg/ml dextran sulfate.

**Endocytosis, Retroendocytosis, and Protein Degradation**

**TABLE I**

| Preparation | Labeled BSA (cpm/ml) | Total BSA (mg/ml) | Cellular radioactivity (cpm/cell$^b$) | Normalized accumulation (μl/mg cells$^a$) |
|-------------|---------------------|-----------------|--------------------------------------|----------------------------------------|
| 1           | 1.7 × 10$^6$        | 0.01            | 531                                  | 0.039                                  |
| 1           | 6.7 × 10$^6$        | 0.08            | 2444                                 | 0.046                                  |
| 1           | 15.0 × 10$^6$       | 0.18            | 5303                                 | 0.044                                  |
| 2           | 4.0 × 10$^6$        | 0.05            | 590                                  | 0.028                                  |
| 2           | 6.0 × 10$^6$        | 0.07            | 866                                  | 0.026                                  |
| 2           | 12.0 × 10$^6$       | 0.15            | 1578                                 | 0.027                                  |
| 2           | 14.0 × 10$^6$       | 0.01            | 1598                                 | 0.023                                  |
| 3           | 14.0 × 10$^6$       | 0.11            | 1500                                 | 0.021                                  |
| 3           | 14.0 × 10$^6$       | 1.11            | 1580                                 | 0.023                                  |

**Part 2. Effect of proteinase inhibitors**

| Inhibitor | Concentration (mM) | Normalized accumulation (μl/mg cells$^a$) |
|-----------|--------------------|----------------------------------------|
| None      |                    | 0.013 ± 0.006                          |
| Antipain  | 0.48               | 0.050 ± 0.017$^d$                      |
| Leupeptin | 0.058              | 0.073 ± 0.012$^d$                      |
| Pepstatin | 0.087              | 0.040 ± 0.010$^b$                      |
| Chloroquine | 0.15               | 0.060$^a$                              |

$^a$ Cellular radioactivity normalized for differences in specific activities and concentrations of the labeled BSA.

$^b$ Average of two determinations.

$^c$ 7.9 × 10$^6$ cells per culture dish.

$^d$ 5.3 × 10$^6$ cells.

$^e$ 5.0 × 10$^6$ cells.

$^f$ Means and standard deviations of three determinations.

$^g$ Difference from control is statistically significant, p < 0.05.

$^h$ Difference is statistically significant, p < 0.01.

steady state between the uptake of BSA and the release of labeled products.

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**Incubation of $^{125}$I-labeled BSA by mouse peritoneal macrophages**

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|-------------|---------------------|-----------------|--------------------------------------|----------------------------------------|
| 1           | 1.7 × 10$^6$        | 0.01            | 531                                  | 0.039                                  |
| 1           | 6.7 × 10$^6$        | 0.08            | 2444                                 | 0.046                                  |
| 1           | 15.0 × 10$^6$       | 0.18            | 5303                                 | 0.044                                  |
| 2           | 4.0 × 10$^6$        | 0.05            | 590                                  | 0.028                                  |
| 2           | 6.0 × 10$^6$        | 0.07            | 866                                  | 0.026                                  |
| 2           | 12.0 × 10$^6$       | 0.15            | 1578                                 | 0.027                                  |
| 2           | 14.0 × 10$^6$       | 0.01            | 1598                                 | 0.023                                  |
| 3           | 14.0 × 10$^6$       | 0.11            | 1500                                 | 0.021                                  |
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$^e$ 5.0 × 10$^6$ cells.

$^f$ Means and standard deviations of three determinations.

$^g$ Difference from control is statistically significant, p < 0.05.

$^h$ Difference is statistically significant, p < 0.01.

increasing substrate concentration at concentrations above the apparent dissociation constant. This conclusion is strongly supported by the observation that increasing concentrations of serum actually enhanced rather than inhibited BSA accumulation, in agreement with earlier findings (34). Part of the differences in the rates and extents of accumulation of labeled BSA and dextran sulfate presumably reflect the ongoing degradation of the former in lysosomes. This is supported by the finding of increased accumulation of BSA in the presence of inhibitors of lysosomal protein degradation (Table I). The much more rapid rate of accumulation of dextran sulfate may also be due to its ability to stimulate pinocytic vesicle formation (40). This is consistent with the observation of a 3-fold increase in the rate of BSA accumulation in the presence of 1 mg/ml dextran sulfate (Fig. 2).

Table II contains the calculated first order rate constants for the pinocytosis of the protein substrates in this study. These were obtained by multiplying the amount of substrate accumulated by macrophages at the steady state by the sum of the degradation and regurgitation rate constants ($k_d + k_r$) in Table III. The calculated rate constants for uptake of all three proteins are essentially identical, providing further support that the mechanism of pinocytosis of these proteins by macrophages lacks a specific adsorptive component.

**Exocytosis and Degradation of Labeled Substrates**—Fig. 3 shows first order plots of the release of trichloroacetic acid soluble and insoluble radioactivity from cultured macrophages after an overnight (24 h) accumulation of labeled BSA. The term $F$, in Fig. 2, corresponds to either $1 - (TCA)_{sol}$/[TCA]$_{insol}$ or $1 - (TCA)_{sol}$/[TCA]$_{insol}$. The points are the means and standard deviations of 27 separate experiments. It is evident that the slopes of the lines for trichloroacetic acid/phosphotungstic acid-soluble and -insoluble radioactivity are similar indicating similar or identical observed rate constants for the two products. These slopes and standard errors were 0.0357 ± 0.0013 h$^{-1}$ and 0.0330 ± 0.0019 h$^{-1}$ for trichloroacetic acid/phosphotungstic acid-soluble and -insoluble products. Similar results were also obtained with enolase and catalase in MPM and with BSA in BHK cells (Table III). These results are consistent with model 1 in which a common substrate pool is the source of both products. From the ratio of trichloroacetic acid/phosphotungstic acid-soluble to total products released, $R$, the intrinsic degradation and regurgitation rate constants, $k_d$ and $k_r$, were calculated as described above. These are also collected in Table III.

**Fig. 4** shows data for BSA plotted according to an alternative method developed for parallel first order reactions in which a common substrate gives rise to two different products (38). The data is derived from a subset of 13 separate experiments chosen at random. In part a, the log of the total radioactivity remaining in the cell is calculated for each time point and plotted against time, as suggested by Equation 3. The slope of the least squares line, 0.0387 ± 0.0046 h$^{-1}$, corresponds to the observed rate constant, $k_{obs}$, for the disappearance of the total intracellular substrate pool. This value

**TABLE II**

| Protein | Normalized cellular accumulation (μl/mg cells$^a$) | $k_{obs}$ (μl/h/mg cells$^a$) |
|---------|-----------------------------------------------|-------------------------------|
| BSA     | 0.380 ± 0.012 (17)                             | 0.65 × 10$^3$                |
| Enolase | 0.078 (2)                                     | 0.34 × 10$^3$                |
| Catalase| 0.045 ± 0.016 (3)                              | 0.50 × 10$^3$                |
Photungstic acid-soluble, product was analyzed separately as described under "Results." The data points are the means of phosphotungstic acid insoluble, calculated by the methods of least squares. The slopes of the straight lines are expected. The slopes of the lines should be equal to that obtained from the slopes of the logarithmic plots in Fig. 3. In part b, the fraction of the initial radioactivity remaining in the cell and appearing in the medium as trichloroacetic acid/phosphotungstic acid-soluble and -insoluble forms are individually plotted against 1 - e^{-kt}. If trichloroacetic acid/phosphotungstic acid-soluble and -insoluble radioactive pool which disappears with rate constant k, then straight lines are expected. The slopes of the lines should be 1.0, k/k, and k/k (38). The actual values obtained were 0.982 ± 0.033, 0.639 ± 0.021, and 0.241 ± 0.053. The corresponding rate constants, k, and k, were calculated to be 0.0247 ± 0.0030 h^{-1} and 0.0082 ± 0.0026 h^{-1}, in good agreement with the values obtained in Table III obtained by the first method.

Fig. 5 shows the Sephadex G-50 elution profiles of media samples obtained after incubating [125I]iodo-BSA-laden BHK cells for 24 h in unlabeled medium. The elution profile for the sample prior to trichloroacetic acid/phosphotungstic acid-precipitation showed the presence of large and small molecular weight components. The peak centered at fraction 12, corresponding to low molecular weight material, appeared to be due to several components. Trichloroacetic acid/phosphotungstic acid treatment selectively removed high and intermediate molecular weight label as expected. Of specific interest is the observation that radiolabeled material released into the medium by cultured fibroblasts was qualitatively similar to that recovered from the whole cell lysate and included intact and partially degraded protein.

The release of trichloroacetic acid/phosphotungstic acid-precipitable radioactivity from cultured cells was too rapid to be due to cell leakage or cell death. This was shown in two ways. When fibroblasts were metabolically labeled for 40 h with L-[4,5-3H]leucine, they were observed to release trichloroacetic acid/phosphotungstic acid-precipitable radioactivity into the medium in a biphasic manner. The initial phase corresponded to 18% of the total trichloroacetic acid/phosphotungstic acid-precipitable radioactivity released during the chase period (about 5% of the total cellular radioactivity) and was characterized by an observed rate constant of 0.33 h^{-1}. The remaining 82% of the trichloroacetic acid/phosphotungstic acid-precipitable radioactivity was released with a rate constant of 0.0053 h^{-1}. Leakage rates were also estimated by
after an overnight incubation with cells. These results are consistent with suggestions that chloroquine rapidly permeates MPM and becomes concentrated in lysosomes (18) while pepstatin is taken up more slowly by pinocytosis (41). They also provide evidence that the degradation of pinocytosed proteins occurred in lysosomes and did not involve the action of secreted proteases on regurgitated proteins. This conclusion was also supported by the observation that neither fresh medium nor medium conditioned by the prior growth of cells were capable of releasing trichloroacetic acid/phosphotungstic acid-soluble products from labeled substrates at measurable rates. Increased serum concentrations, chloroquine, and pepstatin also caused an increase in the proportion of the substrate released into the medium in a trichloroacetic acid/phosphotungstic acid-precipitable form, and was reflected in decreases in the value of R (Table IV).

**Discussion**

It has previously been observed that both macrophages and fibroblasts release a variety of nondigestible solutes following their uptake by endocytosis (23, 24). Polypeptide fragments of intact proteins may also be secreted as a consequence of antigen (3) or peptide hormone (42) processing by macrophages. Fibroblasts have been shown to regurgitate 125I-labeled low density lipoproteins following receptor-mediated uptake (25), and studies of the receptor-dependent metabolism of transferrin in mouse teratocarcinoma stem cells (26) and asialoglycoproteins in rat hepatocytes (27), and the fluid phase uptake and metabolism of horseradish peroxidase in Chinese hamster ovary cells (43) and hemoglobin A in cultured chick myotubes (44) suggest that this may be a general phenomenon. These reports contradict earlier claims that there is no consistent release of intact proteins or trichloroacetic acid-precipitable products following fluid phase pinocytosis (28). It has also been argued that to the extent that regurgitation might occur, it solely involves the participation of endosomes (29, 30, 43) and is a necessary consequence of endosomal membrane recycling via small vesicles containing an entrapped aqueous space (29). In this work we have consistently observed the release of 20–30% of accumulated substances as trichloroacetic acid/phosphotungstic acid-precipitable radioactive activity following fluid phase pinocytosis of proteins. This material consisted of intact protein as judged by gel exclusion chromatography and SDS-polyacrylamide gel electrophoresis as well as intermediate molecular weight fragments, and was qualitatively similar to the labeled material recovered from whole cell lysates.

Strong circumstantial evidence exists for the involvement of endosomes in substrate regurgitation. Following uptake, 20–40% of the protein substrate rapidly reappears in the medium in a trichloroacetic acid-insoluble form. This rapid exocytosis is largely complete by 60 min and has a half-life of about 5–10 min (26, 27, 43, 44). This is followed by a slower loss of label from the cells and the appearance of trichloroacetic acid-soluble products (43). In this present work, we do not dispute the importance of pinocytic vesicle recycling in substrate regurgitation. However, we present evidence that the slow phase of disappearance of labeled substrates from MPM and BHK cells (t > 10 h) is also accompanied by the appearance of 20–30% of the remaining label as trichloroacetic acid/phosphotungstic acid-precipitable material. Furthermore, we have shown that the half-life of the substrate pool which gives rise to these trichloroacetic acid/phosphotungstic acid-precipitable products at late times is identical to the half-life of the substrate pool from which trichloroacetic acid/phosphotungstic acid-soluble products are derived. As a consequence, the

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**Table IV**

**Effect of proteinase inhibitors and serum on BSA degradation and regurgitation in macrophages and fibroblasts**

| Inhibitor | Concentration | TCA/PTA* soluble | TCA/PTA insoluble | R |
|----------|--------------|-----------------|-----------------|---|
| None     | mM           | h⁻¹             |                 |   |
| Chloroquine (2) | 0.156       | 0.0069          | 0.0084          | 0.37 |
| Pepstatin (2) | 0.098       | 0.0154          | 0.0148          | 0.63 |
| Serum (2) |                | 0.0320          | 0.0308          | 0.77 |

* TCA/PTA, trichloroacetic acid/phosphotungstic acid.

Numerical determinations.

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**Figure 5.** Molecular exclusion chromatography of labeled medium samples and cell extracts. Samples were obtained after incubating [125]iodo-BSA-laden fibroblasts in unlabeled medium for 24 h. The column bed, 1.5 x 30 cm, was equilibrated and eluted with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl. [●] release medium before treatment with trichloroacetic acid/phosphotungstic acid; [O], supernatant fraction following trichloroacetic acid-phosphotungstic acid treatment of the release medium; [●], an untreated fibroblast extract.

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**Inhibition of Protein Degradation**—Table IV shows the effects of increased serum concentration, pepstatin, and chloroquine on the metabolism of one preparation of BSA by cultured MPM and BHK cells. Addition of 50% serum to the chase medium caused a 35% decrease in kₘ for both degradation and regurgitation. Chloroquine and pepstatin were also seen to decrease kₘ for the release of trichloroacetic acid/phosphotungstic acid-soluble and insoluble radioactivity. However, chloroquine inhibited when added directly to the release medium whereas pepstatin inhibition was maximum measuring the levels of lactic dehydrogenase at hourly intervals in media harvested from cultured fibroblasts. When divided by the level of lactic dehydrogenase recovered from the corresponding cell homogenates, the fractional leakage rate was calculated to be 0.0042 h⁻¹. These rate constants were significantly smaller than the value of 0.0250 h⁻¹ obtained for kₘ for the release of trichloroacetic acid/phosphotungstic acid insoluble radioactivity arising from endocytosed proteins in fibroblasts (Table IV).
appearance of trichloroacetic acid/phosphotungstic acid-precipitable radioactivity parallels that of trichloroacetic acid/phosphotungstic acid-soluble radioactivity. The simplest explanation of this finding is that both products arise from the same intracellular substrate pool. The failure of the two pool-two product models to describe degradation and exocytosis in this present study strongly argues against the endosome as the exclusive source of regurgitated proteins in cultured MPM and BHK cells. Indeed, the prolonged 30-min wash period which we employed to remove possible surface adsorbed proteins should have insured that the endosome recycling phase of regurgitation would have been largely over by the beginning of the observation period. Our failure to detect a second pool of labeled substrate which could give rise to trichloroacetic acid/phosphotungstic acid-precipitable radioactivity also strongly argues against desorption of substrates from the surface of cells or the culture dish as the source of this material. Additional observations which support these conclusions are as follows. Increasing concentrations of BSA and serum in the uptake medium failed to decrease the cell associated radioactivity or reduce the amount of trichloroacetic acid/phosphotungstic acid-precipitable radioactivity released during the subsequent chase. Blank plates preincubated with labeled substrates for 24 h in the presence and absence of serum were subsequently observed to release an amount of label which was usually much less than 5% and 25% of that accumulated by MPM and BHK cells, respectively, during this same period. Chloroquine and pepstatin and 50% fetal calf serum increased the absolute amount of trichloroacetic acid/phosphotungstic acid-precipitable material released by MPM and BHK cells while decreasing $k_{obs}$. Nearly half the trichloroacetic acid-precipitable radioactivity released by BHK cells were of sizes intermediate between that of intact BSA and its acid soluble products, implying that intracellular proteolysis had occurred prior to release.

In order to conclude that a bidirectional vesicular traffic between lysosomes and the plasma membrane is responsible for exocytosis of trichloroacetic acid/phosphotungstic acid-precipitable radioactivity by MPM and BHK cells, it is necessary to first exclude a number of alternative explanations. These explanations are cell death, detachment, leakage, and vesicular shedding. Cell detachment and vesicular shedding are unlikely to have been important because prior centrifugation of the release media at 10,000 $\times$ g and 105,000 $\times$ g failed to significantly reduce the amount of trichloroacetic acid/phosphotungstic acid-precipitable radioactivity in these samples. The results of experiments with fibroblasts metabolically labeled for 40 h with tritiated leucine also argues against cell leakage and cell death as the source of trichloroacetic acid/phosphotungstic acid-precipitable radioactivity. Metabolically labeled fibroblasts were indeed observed to release trichloroacetic acid/phosphotungstic acid-insoluble label. However, the time course for this reaction was quite different from that observed for the regeneration of endocytosed proteins. First, logarithmic plots of the data obtained with metabolically labeled fibroblasts appeared biphasic. When a log peeling procedure was used to analyze these curves, it was found that the initial phase corresponded to less than 18% of the total trichloroacetic acid/phosphotungstic acid-precipitable radioactivity released and represented only 5% of the total cell associated radioactivity. The rate constant for this phase was 0.33 h$^{-1}$. The remaining 82% of the trichloroacetic acid/phosphotungstic acid-precipitable radioactivity (23% of the cell associated radioactivity) was released even more slowly, with a rate constant of 0.0053 h$^{-1}$. In addition, measurements of lactate dehydrogenase levels in media samples yielded a similar value of 0.0042 h$^{-1}$ for the fractional rate of leakage in fibroblasts. In contrast, regeneration of endocytosed proteins by fibroblasts resulted in the appearance of 20–30% of the cell associated radioactivity in a trichloroacetic acid/phosphotungstic acid-precipitable form with a rate constant of 0.0230 h$^{-1}$. A reasonable interpretation of these results is that no more than one-fourth of the trichloroacetic acid/phosphotungstic acid-precipitable label released by fibroblasts following endocytosis could have been due to cell leakage or cell death.

The conclusion that proteins transferred to lysosomes can undergo exocytosis in competition with degradation is further strengthened by observations relating to the effects of inhibitors of lysosomal protein degradation. Thus, 50% fetal calf serum, pepstatin, and chloroquine each caused an increase in the total amount of trichloroacetic acid/phosphotungstic acid-precipitable radioactivity released by the cells, thereby resulting in a decrease in the ratio of trichloroacetic acid-soluble to total radioactivity released ($R$ in Table IV). This is predicted by the one compartment model, since $R$ is equal to the ratio $k_d/(k_r + k_d)$. Thus, a decrease in the intrinsic degradation rate constant, $k_d$, should cause a decrease in $R$. Furthermore, we observed that inhibitors of lysosomal protein degradation actually decreased the observed rate constant which characterized the appearance of trichloroacetic acid/phosphotungstic acid-precipitable radioactivity. This result is also expected since $k_{obs}$ for regurgitation should be equal to the sum of the intrinsic degradation and regurgitation rate constants ($k_d + k_r$), according to the proposed model. Consequently, a decrease in $k_d$ should be reflected in a decrease in $k_{obs}$ for regurgitation as well as for degradation.

The kinetic measurements described in this report are straightforward, and the arguments and conclusions based on these measurements are quite strong. Pulse-chase experiments of the type we described can readily detect the presence of multiple, kinetically different substrate pools. In cases where more than one product is formed, independent analyses of the time course for the appearance of each of the products can permit the assignment of particular products to particular, kinetically distinct pools. Consequently, the observation that the progress curves for the release of trichloroacetic acid/phosphotungstic acid-soluble and -insoluble radioactivity are characterized by identical apparent first order rate constants with each of three different radiolabeled protein substrates provides important kinetic evidence that both products originate from a common intracellular compartment. Since the trichloroacetic acid/phosphotungstic acid-insoluble product included intermediate sized fragments, this compartment is likely to be a degradative compartment, and it is reasonable to conclude that it is the lysosome. However, it should be noted that kinetic measurements do not provide direct evidence for the involvement of lysosomes in substrate regurgitation. In addition, it remains to be established whether the ability of macrophages and fibroblasts to regurgitate intact proteins and partially proteolyzed products is an artifact of cell culture or an important physiological property of these cells in vivo. An important implication of this study is that these cells may have the ability to modify their external environment by internalization, partial proteolysis, and exocytosis of extracellular proteins. A similar pathway has been described for antigen presentation by macrophages and for the generation of active fragments of parathyroid hormone by macrophages and Kupffer cells (42). The finding that fibroblasts can also regurgitate intact proteins and partially proteolyzed products suggest that this property may be more widely distributed.
APPENDIX

Rate equations for model 2 of endocytosis, degradation, and regurgitation depicted in Fig. 1 are as follows.

\[
d(\text{Pe})/dt = -(k_2 + k_3)\text{Pe}
\]

(1)

\[
d(\text{Pl})/dt = k_1(\text{Pe}) - k_2(\text{Pl})
\]

(2)

\[
d([\text{TCA}]_{\text{mol}})/dt = k_2(\text{Pl})
\]

(3)

\[
d([\text{TCA}]_{\text{mol}})/dt = k_1(\text{Pe})
\]

(4)

In these equations it is assumed that step \( k_2 \) in Fig. 1, regurgitation of the contents of lysosomes, is insignificant. Integrating Equation 1 between \( t = 0 \) and \( t = \infty \) yields

\[
[\text{Pe}]_t = [\text{Pe}]_0 e^{-k_2 t}
\]

(5)

Combining Equation 5 with Equation 4 and integrating between \( t = 0 \) and \( t = \infty \) gives

\[
[\text{[TCA]}_{\text{mol}}] = -[k_1\text{Pe}_0/(k_2 + k_3)]e^{-k_2 t} + C
\]

(6)

Evaluating \( C \) at \( t = 0 \) gives \( C = k_1\text{Pe}_0/(k_2 + k_3) \). Substituting for \( C \) in Equation 6 yields

\[
[\text{[TCA]}_{\text{mol}}] = (k_1\text{Pe}_0/(k_2 + k_3))[1 - e^{-k_2 t}]
\]

(7)

Combining Equation 5 with Equation 2 gives

\[
d(\text{Pl})/dt = k_1\text{Pe}_0 e^{k_1 t} - k_2(\text{Pl})
\]

(8)

Rearranging the terms in Equation 8 and multiplying both sides by \( e^{k_1 t} \) yields

\[
d(\text{Pl})/dt\cdot e^{k_1 t} + k_2(\text{Pl})\cdot e^{k_1 t} = k_1\text{Pe}_0 e^{k_1 t} - k_2(\text{Pl})
\]

(9)

The left-hand term in Equation 9 is simply \( d(\text{Pl})/dt\cdot e^{k_1 t} \) and the right-hand term is \( k_1\text{Pe}_0 e^{k_1 t} - k_2(\text{Pl})\). Substituting these into Equation 9 and integrating yields

\[
(\text{Pl})\cdot e^{k_1 t} = k_1\text{Pe}_0 e^{k_1 t}[1 - (k_2 + k_3)](1 - e^{-k_2 t}) + C
\]

(10)

The constant of integration can be evaluated at the limit of \( t = 0 \) where \( \text{Pl}_0 = k_1\text{Pe}_0/[k_2 + (k_3)] \) has the value \( \text{Pl}_0 = k_1\text{Pe}_0/[k_2 + (k_3)] \). Substituting this expression for \( C \) in Equation 10 and dividing both sides by \( e^{k_1 t} \) yields

\[
[\text{Pl}]_t = [\text{Pl}]_0 - k_1\text{Pe}_0/[k_2 + (k_3)] + (k_1\text{Pe}_0/[k_2 + (k_3)])e^{-k_2 t}
\]

(11)

Combining Equation 11 with Equation 3 and integrating yields.

\[
[\text{[TCA]}_{\text{mol}}] = -([\text{Pl}]_0 - k_1\text{Pe}_0/[k_2 + (k_3)])e^{-k_2 t}
\]

(12)

Again evaluating \( C'' \) at the limit of \( t = 0 \) where \( [\text{[TCA]}_{\text{mol}}] \) yields \( C'' = [\text{Pl}]_0 - k_1\text{Pe}_0/[k_2 + (k_3)] + k_2k_1\text{Pe}_0/[k_2 + (k_3)] \). Substituting for \( C'' \) in Equation 12 yields the following.

\[
[\text{[TCA]}_{\text{mol}}] = ([\text{Pl}]_0 - k_1\text{Pe}_0/[k_2 + (k_3)])(1 - e^{-k_2 t})
\]

(13)

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