The Effects of Basic Substances and Acidic Ionophores on the Digestion of Exogenous and Endogenous Proteins in Mouse Peritoneal Macrophages

Shoji Ohkuma, Jerzy Chudzik, and Brian Poole

The Rockefeller University, New York, New York 10021. Dr. Ohkuma's present address is Department of Microbiology and Molecular Pathology, Faculty of Pharmaceutical Sciences, Tokyo University, Sagamiko, Kanagawa 199-01, Japan. Dr. Chudzik's present address is Department of Clinical Biochemistry, University of Toronto, Toronto M5G 1L5, Canada.

Abstract. Basic substances and acidic ionophores that increase the lysosomal pH in cultured macrophages (Ohkuma, S., and B. Poole, 1978, Proc. Natl. Acad. Sci. USA., 75:3327-3331; Poole, B., and S. Ohkuma, 1981, J. Cell Biol., 90:665-669) inhibited the digestion of heat-denatured acetylated bovine serum albumin (BSA) taken up by the cells. For several substances, the shift in pH sufficed to explain the inhibition of proteolysis. Additional effects, presumably on enzyme activities, have to be postulated for tributylamine, amantadine, and chloroquine. Sodium fluoride (10 mM) had no significant effect on the breakdown of BSA by macrophages.

The breakdown of endogenous macrophage proteins, whether short lived or long lived, was inhibited ~40% by 10 mM NaF and 30%, or sometimes less in the case of long-lived proteins, by 100 μM chloroquine. When the cells were supplied with BSA, a mixture of cell proteins, or even inert endocytosible materials, the breakdown of endogenous long-lived proteins and the inhibitory effect of chloroquine on this process were selectively reduced. Inhibition of endocytosis by cytochalasins B or D did not affect the chloroquine-sensitive breakdown of endogenous proteins, indicating that the proteins degraded by this process were truly endogenous and not taken in from the outside by cellular cannibalism. On the other hand, when macrophage proteins were supplied extracellularly, their breakdown occurred at the same rate for short-lived and long-lived proteins, and it was strongly inhibited by chloroquine and not by NaF.

It is concluded from these results that the breakdown of endogenous proteins, both short-lived and long-lived, probably takes place partly (~30%) in lysosomes and partly through one or more nonlysosomal mechanism(s) unaffected by chloroquine and presumably susceptible to inhibition by fluoride. A difference must exist between short-lived and long-lived proteins in the manner in which they reach lysosomes or are handled by these organelles; this difference would account for the selective effect of the supply of endocytosible materials on the lysosomal processing of long-lived proteins.

Materials and Methods

Materials

L-[4,5-3H]Leucine (6 Ci/mmole) was purchased from Schwarz/Mann (Orangeburg, NY), L-[1-14C]Leucine (20 mCi/mmole) was from New England Nuclear (Boston, MA), and [3H]acetic anhydride was from ICN (International Chemical & Nuclear Corp., Irvine, CA). Chloroquine diphosphate and gramicidin were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was obtained from Flow Laboratories, Inc. (McLean, VA). Valinomycin and carbonyl cyanide m-chlorophenyl hydrazone were from Calbiochem-Behring Corp. (La Jolla, CA). Concanavalin A was obtained from Miles Laboratories, Inc., Elkhart, IN. Cytochalasin D was a gift from Dr. H. Minato, Shionogi Research Laboratory, Osaka, Japan. Cytochalasin B was obtained from Imperial Chemical Industries Research Laboratories (Macclesfield, Cheshire, U.K.).

Cell Cultures

Our procedure for the culture of peritoneal macrophages in Dulbecco's minimum essential medium supplemented with 20% fetal bovine serum, adapted from that of Cohn and Benson (5), has been described previously (14, 15). P388D1 cells were a gift from Dr. S. Gordon (now at Sir William Dunn School of Pathology, Oxford, UK) and were grown in suspension. The cell viability was assessed by the Trypan blue dye exclusion method.

Preparation of Labeled BSA

Bovine serum albumin (BSA) (from Miles Laboratories Inc.) was acetylated with [3H]acetic anhydride by the method of Montellaro and Rupert (12) to a...
Density of 1 to 2 acetyl residues per molecule of BSA. The labeled BSA, at a concentration of 5 mg/ml, in Dulbecco's phosphate-buffered saline without dipotassium hydrogen phosphate (PBS(-)) (11) for cell-free experiments or in medium for cell culture experiments, was heat coagulated at 100°C for 5 min and then dispersed vigorously in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,500 rpm for 10 min to eliminate large aggregates and the supernatant was dialyzed extensively against PBS(-) or medium as required.

**Preparation of Labeled Macrophages**

Endogenous macrophage proteins with long and short half-lives were labeled selectively by the technique used by Poole and Wibo (17) for fibroblasts. For the labeling of long-lived proteins, the cells were incubated for 24 h in the presence of 10 μCi (2 μCi/ml) labeled BSA, washed four times with Hanks′ balanced salt solution, and reincubated for 5-16 h to allow the short-lived [3H]-labeled proteins to decay. The cells were then washed and either used as such or re-incubated for 1 h with [3H]leucine (0.6-1.0 mg/ml) to label the short-lived proteins, washed, and then used immediately.

Cells to be used as substrate were cultured in Roux bottles (150 cm2 growth surface, 50 ml medium) and labeled as just described. After washing, the labeled cells were killed by freezing at the temperature of dry ice/acetone. Then they were thawed, scraped from the glass surface with a rubber policeman into medium containing 20% fetal bovine serum, and suspended in a Dounce homogenizer with loose-fitting pestle.

**Measurement of Protein Breakdown by Macrophages**

For studies of the degradation of exogenous proteins, the cells were incubated for 2 h in the presence of labeled BSA (0.6-1.0 mg/ml) or of disrupted labeled cells (0.1-0.3 mg protein/ml). The cells were then washed four times with Hanks′ balanced salt solution and re-incubated in fresh medium for periods of up to 2 h. Total radioactivity and radioactivity soluble in 8% trichloroacetic acid (TCA-soluble) were measured on samples of the medium and of the cells (solubilized in 0.1 M NaOH-0.4% sodium deoxycholate) taken at the beginning and end of the final incubation. The increase in TCA-soluble radioactivity during incubation provided a measure of the protein digested. It was expressed as percent of the amount of exogenous protein stored by the cells (initial TCA-insoluble radioactivity). The breakdown of endogenous proteins in labeled cells was measured similarly from the increase in TCA-soluble radioactivity during incubation.

All experiments were performed in duplicate (occasionally more). Results are expressed as means ± SD. Confidence levels of observed differences were estimated from the t value.

**Measurement of Cell-free Proteolysis**

Macrophages cultured for 40 h in Roux bottles were scraped off the glass with a rubber policeman and collected in ice-cold 0.25 M sucrose containing 2 mM EDTA (pH 7.4). The suspension was homogenized in a Dounce homogenizer (tight-fitting pestle, 150 strokes) and the post-nuclear supernatant was obtained by centrifugation at 1,500 rpm for 10 min in an International centrifuge. Samples of this preparation were incubated at 37°C under constant shaking for 30 min to 2 h in a total volume of 0.7 ml containing 0.4 mg/ml labeled BSA, 180 μg/ml macrophage proteins, 0.1 M buffer, 1.3 mM 2-mercaptoethanol, and 0.1% Triton X-100. The reaction was stopped by addition of TCA to a final concentration of 5% and 1 mg of BSA as carrier, the mixture was cooled to 0°C, and the radioactivity of the cold TCA-soluble fraction was determined.

**Results**

**Degradation of BSA**

Fig. 1 shows the time course of release of TCA-soluble radioactivity from macrophages previously exposed for 2 h to 1 mg/ml heat-denatured [3H]acetylated BSA. The appearance of degradation products in the medium (Fig. 1A) resulted from a combination of release of pre-formed products from the cells (Fig. 1B) and further breakdown of stored protein. Net digestion (Fig. 1C) during the experimental period amounted to some 30% of the total cellular radioactivity, or 40% of the TCA-insoluble material initially present in the cells. This process, which, for obvious reasons, may be as-
Table I. Effects of Basic Substances on the Digestion of BSA by Macrophages (Exp. 1)

| Compound            | Digestion* |
|---------------------|------------|
| None (Control)      | 100.0 ± 0.5|
| Chloroquine (100 μM)| 90 ± 4.0   |
| NH₄Cl (10 mM)       | 46.2 ± 0.4* |
| NH₄Cl (5 mM)        | 66.0 ± 2.9* |
| Methyamine (10 mM)  | 100.0 ± 6.7 |
| Methyamine (5 mM)   | 34.3 ± 2.5* |
| Dimethylamine (10 mM)| 93.9 ± 4.0 |
| Tris(methylamine) (10 mM)| 33.6 ± 8.8* |
| Ethylamine (10 mM)  | 40.2 ± 0.6* |
| Ethylamine (5 mM)   | 62.9 ± 0.7* |
| Diethylamine (10 mM)| 35.6 ± 0.2* |
| Diethylamine (5 mM) | 58.1 ± 2.5* |
| Triethylamine (10 mM)| 47.2 ± 7.1* |
| Triethylamine (5 mM) | 65.4 ± 3.6* |
| N-Butylamine (1 mM) | 88.4 ± 1.6  |
| Tri-n-butylamine (1 mM)| 52.4 ± 8.4* |

* Percentage of the control in which 57.0 ± 0.3% of the BSA taken up was digested (means ± SD of duplicates).

Table II. Effects of Basic Substances on the Digestion of BSA by Macrophages (Exp. 2)

| Compound            | Digestion* |
|---------------------|------------|
| None (Control)      | 100.0 ± 1.0|
| Chloroquine (100 μM)| 13.4 ± 11.5 |
| NH₄Cl (10 mM)       | 45.4 ± 0.0* |
| Methyamine (10 mM)  | 41.1 ± 9.1 |
| Aniline (10 mM)     | 67.7 ± 5.4* |
| Pyridine (10 mM)    | 81.4 ± 0.7* |
| Imidazole (10 mM)   | 85.0 ± 2.3* |
| Amanadine (500 μM)  | 62.9 ± 1.0 |
| Ephedrine (1 mM)    | 69.7 ± 0.7 |
| Atropine (500 μM)   | 51.6 ± 3.6 |
| Mecamylamine (500 μM)| 68.8 ± 1.3* |
| Eserine (1 mM)      | 59.8 ± 6.4 |
| Procaine (1 mM)     | 59.5 ± 6.0 |
| Lidocaine (1 mM)    | 84.0 ± 8.9 |
| Quinine (100 μM)    | 46.6 ± 2.2* |
| Propranolol (100 μM)| 42.8 ± 4.2 |
| Tetraethylammonium chloride (10 mM)| 108.1 ± 11.4 |
| Concanavalin A (50 μg/ml)| 104.9 ± 1.2* |
| Sucrose (80 mM)     | 152.4 ± 8.9* |

* Percentage of the control in which 20.9 ± 1.4% of the BSA taken up was digested (means ± SD of duplicates).

Figure 2. Effects of basic substances and ionophores on the intralysosomal pH (from references 14 and 16) and on the digestion of BSA (from Tables I and III) in macrophages. O, no addition; X, NH₄Cl (1, 5, and 10 mM); ○, methylamine (1, 5, and 10 mM); □, triethylamine (5 and 10 mM); ▽, chloroquine (100 μM); △, amantadine (0.5 mM); ■, tributylamine (1 mM); □, carbonyl cyanide m-chlorophenyl hydrazine (200 μM); ▼, nigericin (5 μg/ml).

natively, they could be due to some artefact of the in vitro system. Whatever the explanation, it seems likely that the in vivo relationship of lysosomal proteolysis to pH is closed to the curve of Fig. 2 and to those observed in vitro with purified lysosomal extracts or enzymes (4, 7) than it is to that of Fig. 3, obtained with crude homogenates. If this is true, then the results of Fig. 2 would indicate that a number of inhibitory compounds exert their effect simply by way of a pH shift. Chloroquine, tributylamine, and amantadine were distinctly more inhibitory than would be expected from their effect on the lysosomal pH and presumably have one or more additional effects. Indeed, chloroquine is known from the work of Wibo and Poole (22) to strongly inhibit cathepsin B at the concentration level it is believed to reach within the lysosomes when present in the medium at 100 μM concentration.
often accompanies the pH shift caused by weakly basic compounds and by proton ionophores, plays no role in the observed inhibition of lysosomal proteolysis. Tributylamine, which does not induce vacuolation and actually inhibits vacuolation by other weakly basic compounds (15), is strongly inhibitory. On the other hand, concanavalin A, which causes vacuolation but only a slow and inconsequential pH shift (15, 16), did not inhibit proteolysis, whereas 80 mM sucrose, which resembles concanavalin A in its effects on vacuolation and lysosomal pH, actually stimulated the breakdown of stored BSA by some unknown mechanism.

It should be noted that the observed effects are not peculiar to the digestion of BSA. A number of the substances tested were found to inhibit similarly the breakdown of other exogenously supplied proteins, including the total macrophage proteins used in some of the experiments described below (see Table VII).

**Digestion of Endogenous Proteins**

As shown by the results of Fig. 4 and Table IV, the breakdown of both short-lived and long-lived endogenous proteins by macrophages was partially inhibited by 100 µM chloroquine and by 10 mM NaF, very much as it is in fibroblasts (2, 17-20, 22). The effect of chloroquine was more variable, and often less marked, than that of fluoride.

When pre-labeled macrophages were given BSA, the rate of degradation of short-lived endogenous proteins was unaffected, but that of the long-lived proteins was decreased (Tables V and VI). The same effect was observed when the cells were allowed to take up other proteins (see below Tables VII and IX), and even indigestible materials, such as polystyrene particles or sucrose (results not shown), as also reported by Dean (8). Loading the cells with BSA did not significantly modify the inhibitory effects of chloroquine or fluoride on endogenous protein breakdown, except, perhaps, for the effect of chloroquine on the degradation of long-lived proteins, which was somewhat less marked. As shown by the results of Table VI and illustrated in Fig. 5, the breakdown of exogenous BSA and that of endogenous proteins (long lived) clearly respond differently to the inhibitors, even when studied simultaneously in the same cells. If chloroquine susceptibility is taken to indicate a lysosomal localization, it would appear that no more than about one-third of the endogenous proteins of macrophages are degraded in lysosomes, a conclusion consistent with previous findings on fibroblasts (2, 17-20, 22). Note also that the experiments were designed so that the cells would degrade comparable amounts of endogenous and exogenous proteins (20-40 µg/mg of macrophage protein). Therefore, unequal loading of the lysosomal system could not account for the observed differences in chloroquine sensitivity.

**Table IV. Effects of Chloroquine and Fluoride on Endogenous Protein Degradation in Doubly Labeled Macrophages**

| Medium          | Short-lived proteins [3H] (n = 3) | Long-lived proteins [14C] (n = 4) |
|-----------------|----------------------------------|----------------------------------|
| Control         | 18.3 ± 1.9 (100)                 | 4.1 ± 0.3 (100)                  |
| 100 µM chloroquine | 12.9 ± 0.4 (70)*               | 2.8 ± 0.3 (68)*                  |
| 10 mM NaF       | 12.0 ± 1.5 (66)*                 | 2.4 ± 0.2 (59)*                  |

Values are means ± SD; n = number of experiments.

* P < 0.001.
† P < 0.01.
‡ P < 0.025.

**Figure 4. Effects of chloroquine and fluoride on endogenous protein degradation in doubly labeled macrophages. (A) Short-lived proteins (3H); (B) long-lived proteins (14C). O, control; ●, 100 µM chloroquine; ■, 10 mM fluoride.
Table II. Effects of Chloroquine and Fluoride and of BSA Feeding on Endogenous Protein Degradation in Doubly Labeled Macrophages

| Medium          | Short-lived proteins | Long-lived proteins |
|-----------------|----------------------|---------------------|
|                 | −BSA                 | +BSA + comparable   |
| Control         | 13.0 ± 0.9 (100)     | 13.3 ± 0.5 (102)    |
| 100 µM chloroquine | 9.3 ± 0.2 (72)**     | 9.5 ± 0.3 (102) (71)** |
| 10 mM NaF       | 7.8 ± 0.7 (60)**     | 7.1 ± 0.5 (91) (53)** |

Doubly labeled cells were incubated with or without unlabeled BSA (12 mg/ml) for 30 min, washed, and re-incubated for measurement of endogenous protein breakdown. BSA uptake (estimated from the resulting decrease in the specific radioactivity of cell proteins) amounted to 0.57 ± 0.08 µg/µg of macrophage protein. Values are means of duplicates ± SD.

* P < 0.005.
* P < 0.01.
* P < 0.025.
** P < 0.05.
Others: P > 0.05.

Table III. Effect of Feeding BSA on the Degradation of Endogenous Long-lived Proteins in Macrophages and Effects of Chloroquine and Fluoride on the Degradation of Endogenous Long-lived Proteins and of Exogenous BSA in the Same Cells

| Medium          | Endogenous Proteins | BSA |
|-----------------|----------------------|-----|
|                 | −BSA                 | +BSA |
| Control         | 2.87 ± 0.11 (100)    | 1.99 ± 0.09 (69)* (100) |
| 100 µM chloroquine | 2.29 ± 0.04 (80)*    | 1.70 ± 0.00 (74)* (85)** |
| 10 mM NaF       | 1.65 ± 0.05 (57)*    | 1.24 ± 0.06 (75)* (62)* |

Cells having their long-lived proteins labeled with [14C]leucine were incubated with or without labeled BSA. Uptake of BSA amounted to 0.130 ± 0.012 µg/µg of macrophage protein. Values are means of duplicates ± SD.

* P < 0.005.
* P < 0.025.
** P < 0.05.
Others: P > 0.05.

Figure 5. Effects of chloroquine and fluoride on the degradation of endogenous long-lived proteins and of BSA in macrophages. (A) BSA; (B) endogenous proteins. O, control; •, 100 µM chloroquine; ■, 10 mM fluoride.

However the possibility still remained that qualitative differences between the substrates (BSA as opposed to a complex mixture of endogenous proteins), rather than the involvement of different degradative systems, might be responsible for the different inhibitor effects. To test this possibility, we repeated the experiments on macrophages that were fed dead macrophages instead of BSA. It is clear from the results of Tables VII and VIII that exogenously supplied macrophage proteins are handled by the cells very much like endocytized BSA, and very differently from the way such proteins are processed in the course of normal turnover. In particular, the rate of breakdown was the same for short- and long-lived proteins when these were exogenously supplied; it was insensitive to fluoride and was strongly depressed by chloroquine. Surprisingly, in these in vivo experiments, we did not observe the greater susceptibility of short-lived proteins to lysosomal...
Table VII. Effect of Feeding Macrophage Proteins on the Degradation of Endogenous Long-lived Proteins in Macrophages and Effects of Inhibitors on the Degradation of Endogenous and Exogenously Supplied Macrophage Long-lived Proteins in the Same Cells

| Medium          | Percent breakdown in 2 h (% of control) | Cells fed dead macrophages |
|-----------------|----------------------------------------|----------------------------|
|                 | Control cells                          | Endogenous proteins       | Exogenous proteins |
| Control         | 4.04 ± 0.15 (100)                      | 2.41 ± 0.10 (60)          | 37.1 ± 1.3 (100)  |
| 100 μM chloroquine | 3.24 ± 0.12 (80)*                       | 2.31 ± 0.01 (71)          | 12.2 ± 0.3 (33)*  |
| 10 mM NaF       | 2.63 ± 0.00 (65)*                       | 1.62 ± 0.02 (62)          | 35.3 ± 0.1 (95)   |
| 10 mM NH₄Cl     | 3.29 ± 0.04 (81)*                       | 2.31 ± 0.05 (70)          | 14.6 ± 0.4 (39)*  |
| 2 μg/ml nigericin | 3.79 ± 0.06 (94)                        | 2.23 ± 0.00 (59)          | 24.9 ± 0.6 (67)*  |
| 20 μg/ml X537A  | 3.96 ± 0.05 (98)                        | 2.42 ± 0.09 (61)          | 24.5 ± 0.4 (66)*  |

Table VIII. Effects of Chloroquine and Fluoride on the Degradation of Endogenous Proteins and of Exogenously Supplied Macrophage Proteins or BSA in Macrophages

| Medium          | Percent breakdown in 2 h (% of control) | Exogenous proteins (fed cells) |
|-----------------|----------------------------------------|-------------------------------|
|                 | Endogenous proteins (control cells) | BSA | Dead macrophage proteins |
|                 | Short-lived | Long-lived | BS | Short-lived | Long-lived | BS |
| Control         | 18.5 ± 1.2 (100) | 4.50 ± 0.10 (100) | 56.1 ± 1.5 (100) | 39.8 ± 2.2 (100) | 39.8 ± 1.6 (100) |
| 100 μM chloroquine | 12.5 ± 1.5 (68)** | 2.89 ± 0.01 (64)* | 8.6 ± 3.7 (15)* | 10.1 ± 0.6 (25)* | 12.2 ± 1.3 (31)* |
| 10 mM NaF       | 11.5 ± 1.2 (62)** | 2.45 ± 0.25 (54)* | 53.3 ± 3.6 (95) | 38.3 ± 0.3 (96) | 36.3 ± 0.1 (91) |

Table IX. Effects of Feeding Dead P388D1 Cells and of Chloroquine and Fluoride on Endogenous Protein Degradation in Doubly Labeled Macrophages

| Medium          | Percent breakdown in 2 h (% of control) | fed P388D1 |
|-----------------|----------------------------------------|------------|
|                 | Short-lived proteins | Long-lived proteins |
|                 | Control Cells | Fed P388D1 | Control Cells | Fed P388D1 |
| Control         | 12.5 ± 1.1 (100) | 11.9 ± 0.6 [95] (100) | 2.70 ± 0.12 (100) | 1.85 ± 0.03 [69] (100) |
| 100 μM chloroquine | 8.7 ± 0.5 (70)** | 8.3 ± 0.9 [95] (70)** | 2.49 ± 0.02 (92) | 2.03 ± 0.08 [82] (110) |
| 10 mM NaF       | 6.2 ± 0.9 (50)* | 7.4 ± 0.5 [119] (62)* | 1.56 ± 0.09 (58)* | 1.16 ± 0.10 [74] (63)* |

Macrophages having their long-lived proteins labeled with [3H]leucine were incubated in a medium containing disrupted macrophages (87 μg of protein/ml) that had been identically labeled with [14C]leucine. The cells, which had taken up 0.063 ± 0.001 μg of dead cell protein/μg of macrophage protein, were washed after 2 h and re-incubated in fresh medium for simultaneous measurement of the breakdown of endogenous and exogenous proteins. Values are means of duplicates ± SD.

Others: P > 0.05.

Table IX. Effects of Feeding Dead P388D1 Cells and of Chloroquine and Fluoride on Endogenous Protein Degradation in Doubly Labeled Macrophages

Doubly labeled macrophages were fed with dead P388D1 cells (2 × 10⁷ cells/ml) for 30 min, washed and reincubated. The amount of P388D1 cell protein taken up (estimated as in Table V) was 0.86 ± 0.08 μg/μg of macrophage protein. Values are means of duplicates ± SD.

Others: P > 0.05.

Though the participation of a nonlysosomal pathway in endogenous protein breakdown seemed clearly established by digestion seen by others in vitro (3, 7, 21). Feeding cells with dead macrophages (Table VII) or with dead P388D1 cells (Table IX) tended to decrease the rate of breakdown of long-lived, but not of short-lived, endogenous proteins to a point where almost no further inhibition by chloroquine could be observed. As already mentioned, a similar effect was seen in cells allowed to endocytose BSA or inert materials. From the decrease in the effect of chloroquine, it would appear that the supply of endocytosible materials affects mostly the lysosomal pathway of processing of endogenous proteins.
Table X. Effects of Cytochalasins and of Chloroquine and Fluoride on the Degradation of Endogenous Long-lived Proteins in Macrophages

| Medium          | Control cells | +Cytochalasin D | Control cells | +Cytochalasin B | +Cytochalasin B |
|-----------------|---------------|-----------------|---------------|-----------------|----------------|
|                 | 1% DMSO       | 10 μg/ml        | 1% DMSO       | 5 μg/ml         | 10 μg/ml       |
| Control         | 3.28 ± 0.01 (100) | 2.92 ± 0.12 [89] | 3.73 ± 0.05 (100) | 2.68 ± 0.08 [71] | 2.58 ± 0.00 (100) |
| 100 mM chloroquine | 2.25 ± 0.05 (60)² | 1.92 ± 0.09 [53]⁴ | 2.72 ± 0.02 (73)² | 1.81 ± 0.17 [67]⑥ | 1.75 ± 0.01 (65)² |
| 10 mM NaF       | 1.89 ± 0.18 (56)² | 1.93 ± 0.01 (102) | 2.01 ± 0.04 (54)² | 1.75 ± 0.01 (87)⑥ | 1.99 ± 0.02 (54)² |
|                 |               |                 |               |                 |                 |

Experiments were performed on macrophages having their long-lived proteins labeled. In Exp. 1 (†), cytochalasin D was present only in the final 24-h incubation period. In Exp. 2 (‡), cytochalasin B was present already in the 16-h washout period, following exposure to [3H]leucine, as well as in the final 24-h incubation period. The cytochalasins were dissolved at 1 mg/ml in DMSO. Final concentration of DMSO was 1% in medium. Values are means of duplicates ± SD.

1 P < 0.001.
1 P < 0.005.
1 P = 0.01.
4 P < 0.025.
** P < 0.05.
Others: P > 0.05.

the preceding results, the alternative possibility now had to be considered that the lysosomal pathway might be spurious and actually concern exogenous proteins taken up as a result of cellular "cannibalism." This possibility was tested with inhibitors of endocytosis. As demonstrated by Table X, cytochalasin D—which in our hands inhibited the uptake of BSA by 93%—had only a marginal effect on the breakdown of long-lived endogenous proteins and did not affect the susceptibility of this process to chloroquine inhibition. Cytochalasin B, which blocked endocytosis less completely, inhibited protein degradation by 30%. However, this effect reduced the fluoride-sensitive part of the process much more than it did the chloroquine-sensitive part. Cannibalism may therefore be excluded as an explanation for the participation of a chloroquine-sensitive pathway in endogenous protein breakdown.

Discussion

The main finding reported in this paper is that the ability of weakly basic compounds and of proton ionophores to raise the lysosomal pH (14, 16) correlates with an inhibitory effect on the lysosomal breakdown of endocytosed proteins. For a number of substances, the pH shift seemed sufficient to account entirely for the observed inhibition. Some compounds exert additional inhibitory effects, presumably at the enzyme level. Chloroquine, which inhibits cathepsin B (22), is a case in point.

Chloroquine also inhibited the breakdown of endogenous proteins. The inhibition was of the order of 30% for short-lived proteins, and of a similar magnitude or less for long-lived proteins. Supplying the cells with BSA or dead cell proteins (or even inert endocytosible materials) had no effect on the breakdown of short-lived proteins or on the sensitivity of this process to chloroquine; but it cut down by 20 to 30% the breakdown of long-lived proteins and reduced the inhibitory effect of chloroquine on this breakdown. If sensitivity to chloroquine is considered indicative of a lysosomal localization, these results suggest that about one-third of both short-lived and long-lived endogenous proteins were broken down in lysosomes and that the supply of exogenous materials decreased the amount of endogenous long-lived proteins broken down in lysosomes (possibly inhibiting their autophagic segregation), but not that of short-lived proteins. Inhibition of endocytosis with cytochalasins had little or no effect on the chloroquine-sensitive breakdown of endogenous (long-lived) proteins, indicating that the proteins broken down by the lysosomal pathway were truly endogenous, and not taken up by some sort of cellular cannibalism. On the other hand, when macrophage proteins were supplied from the outside, short- and long-lived proteins were broken down at the same rate and this process was strongly inhibited by chloroquine (though possibly less strongly than was the breakdown of BSA), making it probable that the very partial inhibition of endogenous protein breakdown by chloroquine was not a substrate-dependent peculiarity of the lysosomal degradation of these proteins, but rather reflected the existence of one or more nonlysosomal pathways of endogenous protein breakdown. The possibility that lysosomes are not involved at all in endogenous protein breakdown and that chloroquine acts on some nonlysosomal mechanism cannot of course be excluded on the basis of our results.

The conclusion that endogenous proteins are probably broken down partly in lysosomes and partly outside these particles is in agreement with previous observations from this laboratory (17-20, 22) and from many others (for reviews see references 1, 10, and 13). However, neither the present findings on macrophages, nor the earlier ones of Wibo and Poole (22) on fibroblasts, support the belief (1, 13) that lysosomes may play a more important role in the degradation of long-lived proteins than in that of short-lived proteins, unless of course chloroquine sensitivity is not exclusive to the lysosomal pathway. A difference must, however, exist in the way the two groups of proteins enter lysosomes or are processed by these organelles, in view of the selective inhibition of the lysosomal breakdown of long-lived proteins by the supply of endocytosible materials. Contrary to in vitro observations by other workers (3, 7, 21) indicating that short-lived proteins are more susceptible to proteolysis than are long-lived proteins, we have found no evidence of such a difference when the proteins are broken down in vivo inside lysosomes.

Fluoride had no effect on the digestion of exogenous proteins and inhibited the breakdown of both short-lived and long-lived proteins by ~40%, whether or not exogenous proteins were supplied. It is tempting to assume that this effect is exerted on some nonlysosomal mechanism, although inhibition of autophagic segregation cannot be entirely excluded. According to results from Poole and Wibo (17) on fibroblasts,
fluoride is not likely to act by reducing the supply of ATP. It could, however, inhibit some ATP-dependent mechanism of protein breakdown or block the action of some calcium-requiring protease (1, 10, 13).

The authors are grateful to Mihoko Ohkuma for expert technical assistance and to Professor C. de Duve (The Rockefeller University) for valuable discussions and for his help in preparing the manuscript.

EDITORIAL STATEMENT: This work was carried out in the laboratory of Brian Poole prior to his untimely death in May of 1981.

Received for publication 28 June 1985, and in revised form 12 November 1985.

References

1. Amenta, J. S., and S. C. Brocher. 1981. Minireview: mechanisms of protein turnover in cultured cells. Life Sci. 28:1195–1208.

2. Amenta, J. S., H. J. Sargus, and F. M. Baccino. 1977. Effect of microtubular or translational inhibitors on general cell protein degradation: evidence for a dual catabolic pathway. Biochem. J. 168:223–227.

3. Bohley, P., C. Miehe, M. Miehe, S. Ansorge, H. Kirschke, J. Langner, and B. Wiederanders. 1972. Intracellular protein degradation. V. Preferential degradation of short-lived cytosol proteins by lysosomal endopeptidases from rat liver. Acta Biol. Med. Germ. 28:323–330.

4. Cuffey, J. W., and C. de Duve. 1968. Digestive activity of lysosomes. I. The digestion of proteins by extracts of rat liver lysosomes. J. Biol. Chem. 243:3255–3263.

5. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes—morphology, cytochemistry and biochemistry. J. Exp. Med. 121:153–170.

6. de Duve, C., T. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. van Hoof. 1974. Commentary: lysosomotropic agents. Biochem. Pharmacol. 23:2495–2531.

7. Dean, R. T. 1975. Lysosomal enzymes as agents of turnover of soluble cytoplasmic proteins. Eur. J. Biochem. 58:9–14.

8. Dean, R. T. 1979. Macrophage protein turnover: evidence for lysosomal participation in basal proteolysis. Biochem. J. 180:339–345.

9. J. T. Finkenstaedt. 1957. Intracellular distribution of proteolytic enzymes in rat liver tissue. Proc. Soc. Exp. Biol. Med. 95:302–304.

10. Hershko, A., and A. Ciechanover. 1982. Mechanisms of intracellular protein breakdown. Annu. Rev. Biochem. 51:335–364.

11. Merchant, D. J., R. H. Kahn, and W. H. Murphy. 1964. In Handbook of Cell and Organ Culture, 2nd ed. Burgess Publishing Co., Minneapolis.

12. Montellaro, R. C., and R. R. Rupert. 1975. Radiolabelling of proteins and viruses in vitro by acetylation with radioactive acetic anhydride. J. Biol. Chem. 250:1413–1421.

13. G. E. Mortimore. 1982. Mechanisms of cellular protein catabolism. Nutr. Rev. 40:1–12.

14. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA. 75:3327–3331.

15. Ohkuma, S., and B. Poole. 1981. Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. J. Cell Biol. 90:656–664.

16. Poole, B., and S. Ohkuma. 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J. Cell Biol. 90:665–669.

17. Poole, B., and M. Wibo. 1973. Protein degradation in cultured cells: the effect of fresh medium, fluoride, and iodoacetate on the digestion of cellular protein of rat fibroblasts. J. Biol. Chem. 248:6221–6226.

18. Poole, B., S. Ohkuma, and M. J. Warburton. 1977. The accumulation of weakly basic substances in lysosomes and the inhibition of intracellular protein degradation. Acta Biol. Med. Germ. 36:1777–1788.

19. Poole, B., S. Ohkuma, and M. Warburton. 1978. Some aspects of the intracellular breakdown of exogenous and endogenous proteins. In Protein Turnover and Lysosome Function. H. L. Segel & D. J. Doyle, editors. Academic Press Inc., New York. 43–58.

20. Poole, B., S. Ohkuma, and M. Warburton. 1980. Protein degradation in cells in culture. Ciba Found. Symp. 75:189–203.

21. Segel, H. L., J. R. Winkler, and M. P. Miyagi. 1974. Relationship between degradation rates of proteins in vitro and their susceptibility to lysosomal proteases. J. Biol. Chem. 249:6364–6465.

22. Wibo, M., and B. Poole. 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B. J. Cell Biol. 63:430–440.