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

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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.

In previous papers, the ability of various basic substances and proton ionophores to induce vacuolation (15) and raise the lysosomal pH (16) in isolated mouse peritoneal macrophages has been described and explained in terms of the proton-trapping model proposed by de Duve et al. (6). The effects of these substances on the intracellular breakdown of exogenous and endogenous proteins are reported in the present paper. Some of these results were presented elsewhere (18–20).

Materials and Methods

Materials

L-[4,5-^{3}H]Leucine (6 Ci/mmol) was purchased from Schwarz/Mann (Orangeburg, NY), L-[1-^{14}C]leucine (20 mCi/mmol) was from New England Nuclear (Boston, MA), and [^{3}H]acetic anhydride was from ICN (International Chemical and 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 [^{3}H]acetic anhydride by the method of Montellaro and Rupert (12) to a

† Deceased.

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 divalent cations (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 [^{14}C]leucine (2 μCi/ml), washed four times with Hanks' balanced salt solution, and reincubated for 15–16 h to allow the short-lived [^{14}C]-labeled proteins to decay. The cells were then washed and either used as such or re-incubated for 1 h with [^{3}H]leucine (20 μCi/ml) to label the short-lived proteins, washed, and then used immediately.

Cells to be used as substrate were cultured in Roux bottles (150 cm^2 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)[1] 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 [^{3}H]acetylated BSA. The appearance of degradation products in the medium (Fig. 1*A*) resulted from a combination of release of pre-formed products from the cells (Fig. 1*B*) and further breakdown of stored protein. Net digestion (Fig. 1*C*) 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 assumed to take place in lysosomes, was unaffected by 10 mM NaF and was inhibited more than 80% by 100 μM chloroquine. Similar results were obtained if the cells were incubated during 30 min after the BSA uptake period to ensure complete phagosome–lysosome fusion before digestion started to be recorded. The inhibition by chloroquine thus clearly affects breakdown in lysosomes, not delivery to lysosomes. Gel filtration on Sephadex G-10 indicated that most of the TCA-soluble radioactivity of both cells and medium belonged to a material behaving like acetyl-lysine.

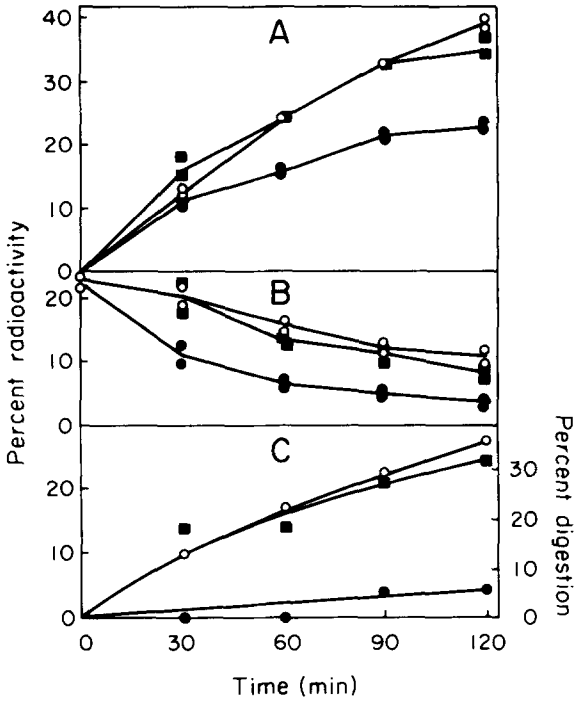

Figure 1. Effects of chloroquine and fluoride on the digestion of BSA by macrophages. (*A*) TCA-soluble radioactivity in the medium; (*B*) TCA-soluble radioactivity in the cells; (*C*) net production of TCA-soluble radioactivity (A + B − [initial TCA-soluble radioactivity in cells]). (*Left ordinate*) Percent of total radioactivity; (*right ordinate*) percent of initial TCA-insoluble radioactivity. ○, control; ●, 100 μM chloroquine; ■, 10 mM NaF. The amount of BSA degraded in 2 h in control medium was 36 μg/mg of macrophage protein.

In a number of experiments which were conducted as that in Fig. 1, macrophages pre-loaded with labeled BSA were exposed to a variety of substances, most of which were previously found to induce vacuolation (15) and/or to raise the intralysosomal pH (14, 16). Under these conditions the cell viability was shown to be more than 90%. As shown by the results listed in Tables I–III, all weakly basic compounds tested, as well as those ionophores that are known to transport protons, inhibited the digestion of BSA significantly. To assess the extent to which the inhibition of proteolysis could be attributed to a rise in lysosomal pH, the two effects were plotted against each other for all compounds whose influence on the lysosomal pH was known from previous work. As illustrated by Fig. 2, most points fall on a curve very similar to those recorded by Coffey and de Duve (4) for the hydrolysis of globin by rat liver lysosomal extracts and by Dean (7) for the breakdown of hepatic cytosol proteins by cathepsins D or B. However, when the influence of pH on the hydrolysis of BSA by macrophage homogenates was investigated, a surpris-

[1] *Abbreviation used in this paper:* TCA, trichloroacetic acid.

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)	9.0 ± 4.0‡
NH_4Cl (10 mM)	46.2 ± 0.4‡
NH_4Cl (5 mM)	66.0 ± 2.9§
NH_4Cl (1 mM)	93.2 ± 0.9¶
Methylamine (10 mM)	34.3 ± 2.2‡
Methylamine (5 mM)	64.8 ± 6.5¶
Methylamine (1mM)	93.9 ± 4.0
Dimethylamine (10 mM)	33.6 ± 8.8‖
Trimethylamine (10 mM)	32.5 ± 3.9§
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 ± 3.1§
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).
‡ $P < 0.001$.
§ $P < 0.005$.
‖ $P < 0.01$.
¶ $P < 0.025$.
Others: $P > 0.05$.

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

Compound	Digestion*
None (Control)	100.0 ± 1.0
Choloroquine (100 μM)	13.4 ± 11.5‖
NH_4Cl (10 mM)	45.4 ± 0.0‡
Methylamine (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¶
Amantadine (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 ± 8.2¶
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 34.3 ± 0.3% of the BSA taken up was digested (means ± SD of duplicates).
‡ $P < 0.001$.
§ $P < 0.005$.
‖ $P < 0.01$.
¶ $P < 0.025$.
** $P < 0.05$.
Others: $P > 0.05$.

ingly different curve was observed (Fig. 3), with two peaks at pH 3.9 and 5.7, and a marked trough at pH 5. The contribution of nonlysosomal proteases or the effect of cathepsin inhibitors (9) could account for these peculiar features. Alternatively, 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.

Our evidence further indicates that vacuolation, which

Table III. Effects of Ionophores on the Digestion of BSA by Macrophages

Compound	Digestion*
None	100.0 ± 6.7
Chloroquine (100 μM)	5.9 ± 2.0§
Methylamine (10 mM)	29.2 ± 1.9§
Nigericin (5 μg/ml)	51.3 ± 5.1¶
X537A (20 μg/ml)	57.7 ± 15.3
Gramicidin (2 μg/ml)	57.6 ± 2.5¶
2,4-Dinitrophenol (1 mM)	82.2 ± 0.7
2,4-Dinitrophenol (10 mM)	68.7 ± 12.4
CCCP (20 μM)	81.8 ± 6.9
CCCP (200 μM)	60.7 ± 8.4**
CCCP (20 μM) + Valinomycin (20 μM)	65.5 ± 5.5**

* Percentage of the control in which 20.9 ± 1.4% of the BSA taken up was digested (means ± SD of duplicates).
CCCP, Carbonyl cyanide *m*-chlorophenyl hydrazone.
§ $P < 0.005$.
¶ $P < 0.025$.
** $P < 0.05$.
Others: $P > 0.05$.

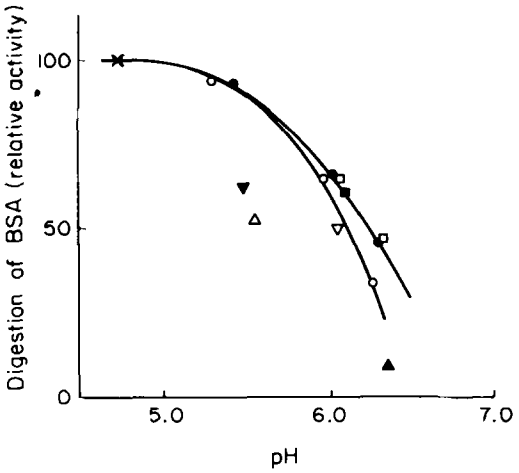

Figure 2. Effects of basic substances and ionophores on the intralysosomal pH (from references 14 and 16) and on the digestion of BSA (from Tables I and III) in macrophages. ×, no addition; ●, NH_4Cl (1, 5, and 10 mM); ○, methylamine (1, 5, and 10 mM); □, triethylamine (5 and 10 mM); ▲, chloroquine (100 μM); ▼, amantadine (0.5 mM); △, tributylamine (1 mM); ■, carbonyl cyanide *m*-chlorophenyl hydrazone (200 μM); ▽, nigericin (5 μg/ml).

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.

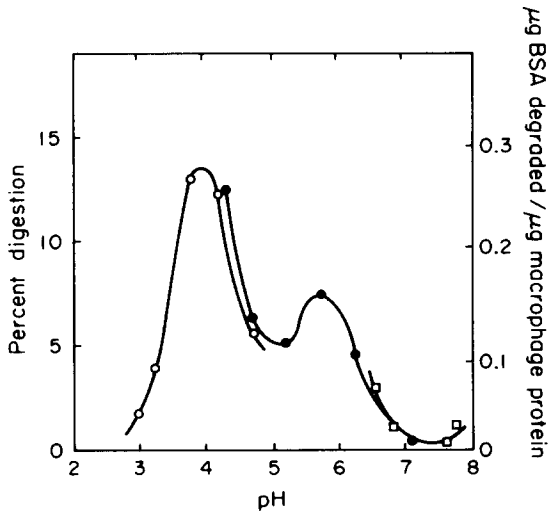

Figure 3. pH-dependence of the digestion of BSA by macrophage extracts. ○, Na-lactate buffer; ●, Na-acetate buffer; □, Na-phosphate buffer.

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

	Percent breakdown in 2 h (% of control)	
Medium	Short-lived proteins [^{3}H] (n = 3)	Long-lived proteins [^{14}C] (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.
‡ $P < 0.001$.
‖ $P < 0.01$.
¶ $P < 0.025$.

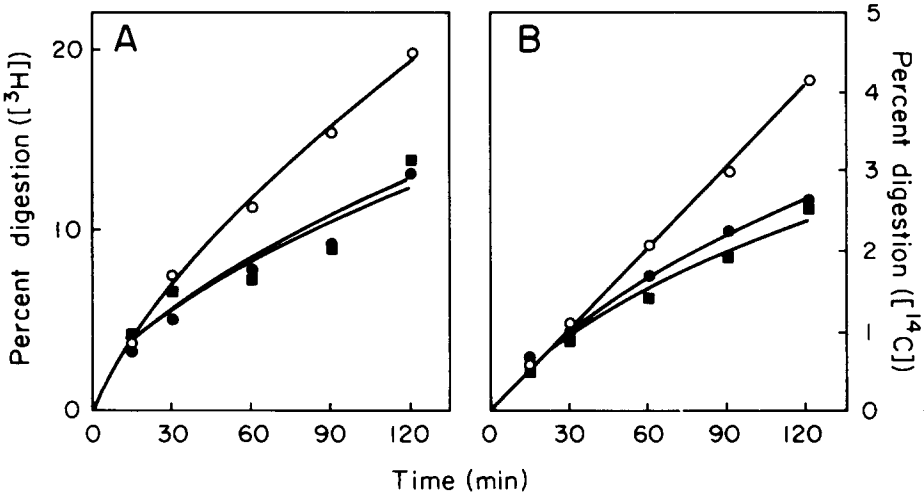

Figure 4. Effects of chloroquine and fluoride on endogenous protein degradation in doubly labeled macrophages. (*A*) Short-lived proteins (^{3}H); (*B*) long-lived proteins (^{14}C). ○, control; ●, 100 μM chloroquine; ■, 10 mM fluoride.

Table V. Effects of Chloroquine and Fluoride and of BSA Feeding on Endogenous Protein Degradation in Doubly Labeled Macrophages

	Percent breakdown in 2 h (% of control) [% of comparable values without BSA]			
	Short-lived proteins		Long-lived proteins	
Medium	−BSA	+BSA	−BSA	+BSA
Control	13.0 ± 0.9 (100)	13.3 ± 0.5 [102] (100)	2.92 ± 0.08 (100)	2.51 ± 0.05 [86]** (100)
100 μM chloroquine	9.3 ± 0.2 (72)**	9.5 ± 0.3 [102] (71)¶	2.35 ± 0.06 (80)¶	2.17 ± 0.03 [92] (86)¶
10 mM NaF	7.8 ± 0.7 (60)¶	7.1 ± 0.5 [91] (53)‖	1.58 ± 0.01 (54)	1.45 ± 0.03 [92]** (58)§

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 VI. 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

	Percent breakdown in 2 h (% of control) [% of comparable values without BSA]		
	−BSA	+BSA	
Medium	Endogenous Proteins	Endogenous Proteins	BSA
Control	2.87 ± 0.11 (100)	1.99 ± 0.09 [69]¶ (100)	32.0 ± 5.5 (100)
100 μM chloroquine	2.29 ± 0.04 (80)¶	1.70 ± 0.00 [74]§ (85)**	6.8 ± 1.8 (21)**
10 mM NaF	1.65 ± 0.05 (57)§	1.24 ± 0.06 [75]¶ (62)¶	31.0 ± 0.0 (97)

Cells having their long-lived proteins labeled with [^{14}C]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: $P > 0.05$.

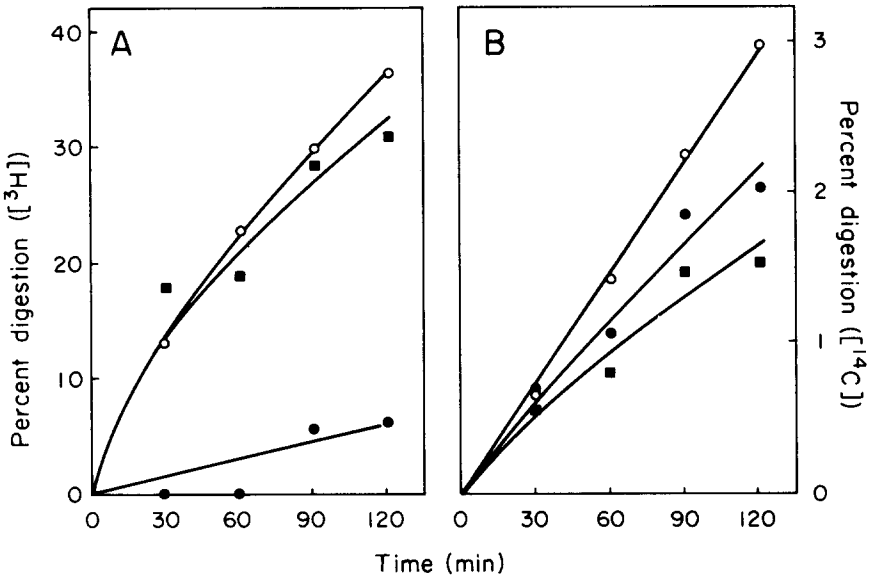

Figure 5. Effects of chloroquine and fluoride on the degradation of endogenous long-lived proteins and of BSA in macrophages. (*A*) BSA; (*B*) endogenous proteins. ○, control; ●, 100 μM chloroquine; ■, 10 mM fluoride.

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

	Percent breakdown in 2 h (% of control) [% of control cells]		
	Control cells	Cells fed dead macrophages	
Medium	Endogenous proteins	Endogenous proteins	Exogenous proteins
Control	4.04 ± 0.15 (100)	2.41 ± 0.10 [60]‖ (100)	37.1 ± 1.3 (100)
100 μM chloroquine	3.24 ± 0.12 (80)¶	2.31 ± 0.01 [71]‖ (96)	12.2 ± 0.3 (33)§
10 mM NaF	2.63 ± 0.00 (65)‖	1.62 ± 0.02 [62]‡ (67)‖	35.3 ± 0.1 (95)
10 mM NH_4Cl	3.29 ± 0.04 (81)¶	2.31 ± 0.05 [70]§ (96)	14.6 ± 0.4 (39)§
2 μg/ml nigericin	3.79 ± 0.06 (94)	2.23 ± 0.00 [59]§ (93)	24.9 ± 0.6 (67)‖
20 μg/ml X537A	3.96 ± 0.05 (98)	2.42 ± 0.09 [61]§ (100)	24.5 ± 0.4 (66)‖

Macrophages having their long-lived proteins labeled with [^{3}H]leucine were incubated in a medium containing disrupted macrophages (87 μg of protein/ml) that had been identically labeled with [^{14}C]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.

‡ $P < 0.001$.

§ $P < 0.005$.

‖ $P < 0.01$.

¶ $P < 0.025$.

Others: $P > 0.05$.

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

	Percent breakdown in 2 h (% of control)				
	Endogenous proteins (control cells)		Exogenous proteins (fed cells)		
				Dead macrophage proteins	
Medium	Short-lived	Long-lived	BSA	Short-lived	Long-lived
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)

In one set (control cells), the breakdown of endogenous proteins in doubly labeled macrophages was measured as in Table V. In the other (fed cells), the breakdown of exogenous proteins was studied in unlabeled macrophages exposed either to labeled BSA (1 mg/ml) or to disrupted doubly labeled macrophages (285 μg protein/ml). The uptake of BSA amounted to 0.05 μg/μg of macrophage protein, that of dead cell protein to 0.097 μg/μg of macrophage protein, with a ratio of long-lived to short-lived proteins (in percent of total) of 1.47. Values are means of duplicates ± SD.

§ $P < 0.005$.

‖ $P < 0.01$.

** $P < 0.05$.

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

	Percent breakdown in 2 h (% of control) [% of control cells]			
	Short-lived proteins		Long-lived proteins	
Medium	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)¶

Doubly labeled macrophages were fed with dead P388D1 cells (2×10^7 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.

‖ $P < 0.01$.

¶ $P < 0.025$.

** $P < 0.05$.

Others: $P > 0.05$.

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 long-lived endogenous proteins.

Though the participation of a nonlysosomal pathway in endogenous protein breakdown seemed clearly established by

Table X. Effects of Cytochalasins and of Chloroquine and Fluoride on the Degradation of Endogenous Long-lived Proteins in Macrophages

Medium	Percent breakdown in 2 h (% of control) [% of control cells]					
	Control cells[1]	+Cytochalasin D[1]		Control cells[2]	+Cytochalasin B[2]	+Cytochalasin B[2]
	1% DMSO	*10 μg/ml*		*1% DMSO*	*5 μg/ml*	*10 μg/ml*
Control	3.28 ± 0.01 (100)	2.92 ± 0.12 [89]	(100)	3.75 ± 0.05 (100)	2.68 ± 0.08 [71]‡	(100)	2.58 ± 0.00 [69]‡	(100)
100 mM chloroquine	2.25 ± 0.05 (69)‡	1.92 ± 0.09 [85]**	(66)¶	2.72 ± 0.02 (73)‡	1.81 ± 0.17 [67]¶	(68)¶	1.35 ± 0.01 [50]‡	(52)‡
10 mM NaF	1.89 ± 0.18 (58)‡	1.93 ± 0.01 [102]	(66)‖	2.01 ± 0.04 (54)‡	1.75 ± 0.01 [87]¶	(65)‖	1.49 ± 0.02 [74]‖	(58)‡

Experiments were performed on macrophages having their long-lived proteins labeled. In Exp. 1 (1), cytochalasin D was present only in the final 2-h incubation period. In Exp. 2 (2), cytochalasin B was present already in the 16-h washout period, following exposure to [^{14}C]leucine, as well as in the final 2-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.

‡ $P < 0.001$.

‖ $P < 0.005$.

‖ $P < 0.01$.

¶ $P < 0.025$.

** $P < 0.05$.

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 nor 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).

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