PROTEIN DEGRADATION IN CULTURED CELLS

II. The Uptake of Chloroquine by Rat Fibroblasts and the Inhibition of Cellular Protein Degradation and Cathepsin B_1

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ABSTRACT

The degradation of cellular proteins in fibroblasts, both those of rapid and those of slow turnover rates, was inhibited by low concentrations of chloroquine or neutral red in the medium. Cells inhibited by chloroquine can be inhibited further by fluoride. Chloroquine was taken up by the fibroblasts and the concentration in the cells reached several hundred times that in the medium. Isopycnic fractionation studies showed that within the cells the chloroquine was concentrated in the lysosomes, and that these chloroquine-containing lysosomes had a lower equilibrium density than the lysosomes of untreated cells. Chloroquine, at concentrations attained inside the lysosomes, inhibited cathepsin B_1 but not cathepsin D. It is concluded that chloroquine impairs the breakdown of cellular proteins after these have entered the lysosome system, probably through inhibition of cathepsin B_1.

Very little is known about the mechanism of degradation of cellular protein during normal protein turnover. It has long been established that the process requires an energy supply (21) and cellular integrity (20). The only known intracellular proteolytic system capable of degrading proteins to their constituent amino acids is in the lysosomes (8). It is well established that some cellular proteins are degraded in lysosomes after the formation of autophagic vacuoles, but the extent to which this process contributes to normal protein turnover is unknown.

The previous paper in this series (18) showed that the degradation of proteins with long half-lives was inhibited in rat fibroblasts by a factor in fresh medium, while the degradation of proteins with short half-lives was unaffected. The degradation of both classes of protein was inhibited by fluoride through some mechanism more specific than the inhibition of glycolysis. In this paper we report the effects of chloroquine.

MATERIALS AND METHODS

Fibroblasts, isolated from trypsinized rat embryos, were cultivated in basal Eagle's medium in culture flasks (75 cm² growth surface, 30 ml medium) or in Leighton tubes (4.8 cm² growth surface, 2 ml medium) and used for experiments between the third and the fifth passage, after confluency was reached. In experiments with labeled media the cells were washed four times with Hanks' solution at each change of medium. At the end of the experiment the washed cells were dissolved in 0.1 M NaOH-0.4% deoxycholate. Total and 8% trichloroacetic acid-soluble radioactivity was measured on each sample of cells and medium as described previously (18). Radio-

activity in the medium is always expressed as a fraction of the total recovered in dissolved cells and medium.

Fibroblasts used for cell fractionation experiments were grown in Roux bottles (150 cm² growth surface, 35 ml medium). They were scraped off the glass surface by means of a rubber policeman and collected in ice-cold 0.25 M sucrose containing 2 mM EDTA pH 7.4. After homogenization in a Dounce grinder (tight-fitting pestle, eight passages), a postnuclear supernate was prepared from the suspension by centrifugation at 6,000 g-min. Isopycnic equilibration of the particles was performed in the Beaufay rotor (14), in which were injected sucessively: 8 ml of postnuclear supernate (4–5 mg protein), 26 ml of a sucrose density gradient extending linearly with respect to volume between densities 1.06 and 1.26, and a 6 ml cushion of sucrose solution with a density of 1.32. The rotor was spun at 35,000 rpm for 35 min. At the end of the run, some 15 fractions were collected from the decelerating rotor (14). The densities were measured by refractometry.

Cathepsin B_1 was measured by the method of Barrett (2). Other enzyme assays were performed as described by Peters et al. (16). The N-acetyl-β-glucosaminidase was found to be inhibited by sucrose. This effect was corrected for in the calculation of the activity of this enzyme. Chloroquine was measured fluorometrically (E 335, A 378). Protein was estimated by the automated Lowry method (14).

Fibroblasts used for phase-contrast microscopy were grown on flying cover slips in Leighton tubes. They were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. The cover slips were then mounted with water and sealed with nail enamel. For electron microscopy, fibroblasts, grown in plastic flasks, were fixed with a mixture of glutaraldehyde and osmium tetroxide as described by Fedorko et al. (9). A few minutes later they were scraped off the surface with a Teflon policeman and further processed as described by Fedorko et al. (9). Sections were stained with lead citrate (19) and examined in a Philips 300 electron microscope at 80 kV.

L-[4,5-³H]Leucine was purchased from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, New York). L-[1-¹⁴C]Leucine was purchased from New England Nuclear (Boston, Mass.). The diphosphate salt of chloroquine was obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

The Effect of Chloroquine and Neutral Red on the Degradation of Cellular Proteins

Fibroblasts growing in flasks were exposed to medium containing [¹⁴C]leucine (1 µCi/ml) for 24 h. Then they were washed and exposed to unlabeled medium for 16 h. During this washout period those proteins with rapid rates of turnover were broken down and resynthesized from unlabeled amino acids, while the proteins with slow turnover rates remained labeled. Then the cells were exposed to medium containing [³H]leucine (10 µCi/ml) for 1 h. During this period the proteins with rapid turnover rates were labeled preferentially. The cells were then washed and placed in unlabeled medium with or without the addition of 100 µM chloroquine. Samples of medium were taken periodically and assayed for radioactivity.

The results of this experiment are shown in Fig. 1. Fig. 1 A shows the output into the medium of trichloroacetic acid-soluble tritium derived from the proteins with rapid turnover rates. From the control cells about 30% of the tritium was released during the period of the experiment. The presence of chloroquine in the medium caused a distinct inhibition of the release of label. In Fig. 1 B we have the results with ¹⁴C. Here, since we have labeled selectively proteins with slow turnover rates, only about 7% of the total label was released from the control cells. Again we have a distinct inhibition of the release of label from the chloroquine-treated cells. This is a true inhibition of proteolysis, since there was no accumulation of trichloroacetic acid-soluble fragments inside the cells. The total radioactivity in the system was the same in the chloroquine-treated cells as it was in the control. No cells were lost by detachment. In Fig. 2 we have plotted the results of an experiment in which cells were labeled with [³H]leucine (1 µCi/ml) for 40 h, followed by an 8-h washout period. The cells were then exposed to unlabeled medium containing 10 or 100 µM chloroquine. After 160 min the medium in all the flasks was removed and replaced either with medium containing chloroquine or with control medium. The higher concentration of chloroquine did not cause much greater inhibition of protein degradation than did the lower one. When the cells were washed and placed in medium without chloroquine (arrow), the inhibition was not reversed.

Table I shows the results of an experiment in which the effects of chloroquine and fluoride on protein degradation were measured alone and together. It is clear from these data that the two types of inhibition were approximately additive.

Table II shows the effect of neutral red on the breakdown of cellular protein. The cellular proteins of slow turnover were labeled with ¹⁴C, and those of rapid turnover were labeled with tritium.
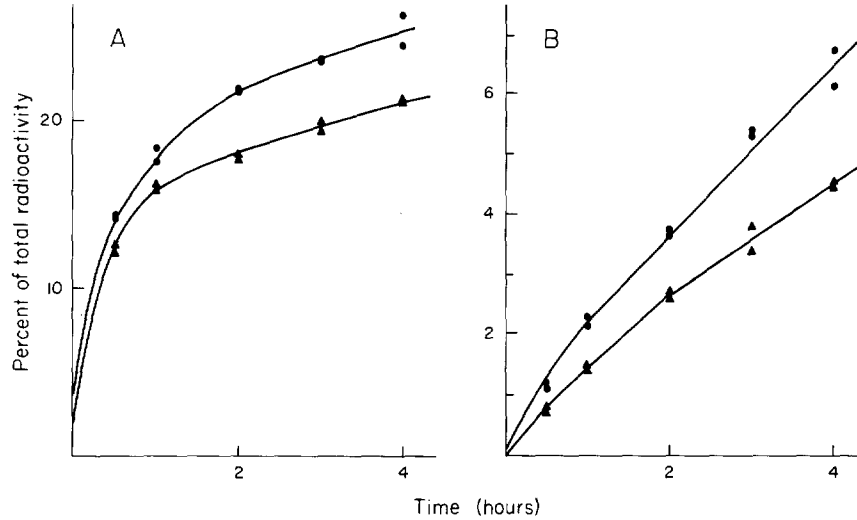
FIGURE 1 The effect of chloroquine on the output into the medium of trichloroacetic acid-soluble label. Fig. 1 A, output of tritium; Fig. 1 B, output of ^{14}C; ●, control; ▲, 100 μM chloroquine.
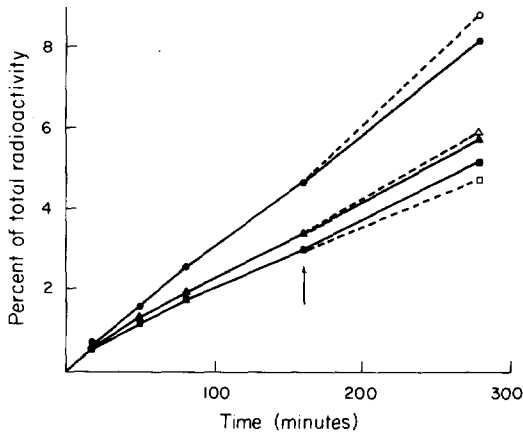

FIGURE 2 Dose dependence and irreversibility of the effect of chloroquine on cellular protein degradation. Curves show output of trichloroacetic acid-soluble tritium into: ●, control medium; ▲, medium containing 10 μM chloroquine; ■, medium containing 100 μM chloroquine. At 160 min (arrow) medium in some tubes was changed to control medium (open symbols, dotted line).

The degradation of both classes of protein was inhibited by 100 μg/ml neutral red to about the same extent as by 100 μM chloroquine.

Morphology of Chloroquine-Treated Cells

Chloroquine at a concentration of 10 or 100 μM induced the appearance of numerous clear vacuoles in the cytoplasm. The effect was faster and

TABLE I

Effects of Chloroquine and Fluoride on Cellular Protein Degradation

Medium	Total isotope trichloroacetic acid soluble in the medium	
	^{3}H	^{14}C
	%	%
Control	22.2 ± 1.4	3.6 ± 0.2
100 μM chloroquine	19.0 ± 1.2	2.7 ± 0.1
10 mM NaF	18.1 ± 1.4	1.9 ± 0.2
100 μM chloroquine + 10 mM NaF	15.9 ± 1.0	1.3 ± 0.1

Cells were labeled for 24 h with [^{14}C]leucine (1 μCi/ml), left in unlabeled medium for 15 h, and then labeled for 1 h with [^{3}H]leucine. Washed cells were incubated in the media shown and the radioactivities were measured after 150 min. Means of 10 ± standard deviation.

more pronounced with the higher dose, but it was somewhat variable from cell to cell. The vacuoles developed in the perinuclear region and by 1–2 h they filled the cytoplasm of most cells. After that, the appearance of the cells remained constant for a few hours. The effect of 100 μM chloroquine is shown in Fig. 3.

After several hours of exposure to 100 μM chloroquine, the cells became detached from the glass. Such was not the case with 10 μM chloroquine. When cells containing vacuoles after expo-

sure to chloroquine were then exposed to medium containing neutral red, these vacuoles stained intensely. The vacuoles did not stain with Oil Red O. When fibroblasts were incubated with chloro-

quine for 2–3 h and then placed in chloroquine-free medium, the vacuoles persisted for at least 3 h.

As shown in Fig. 4 the vacuoles produced by chloroquine were predominantly electron lucent. Some of them contained membrane whorls or smaller vesicles. Peripheral dilatations of the Golgi cisternae were frequent and several images suggested a possible involvement of the Golgi complex in the production of the vacuoles (Figs. 4 and 5). Morphometric measurements performed on electron micrographs of fibroblasts exposed to 100 μM chloroquine for 2 h indicate that the clear vacuoles occupy some 25% of the volume of these cells.

The Uptake of Chloroquine by Fibroblasts

Fig. 6 shows the time-course of chloroquine uptake by fibroblasts growing in Leighton tubes. The initial concentration of the drug in the medium was 100 μM. Uptake had almost stopped after 2 h. When the cells were placed in chloroquine-free medium after 105-min exposure to the
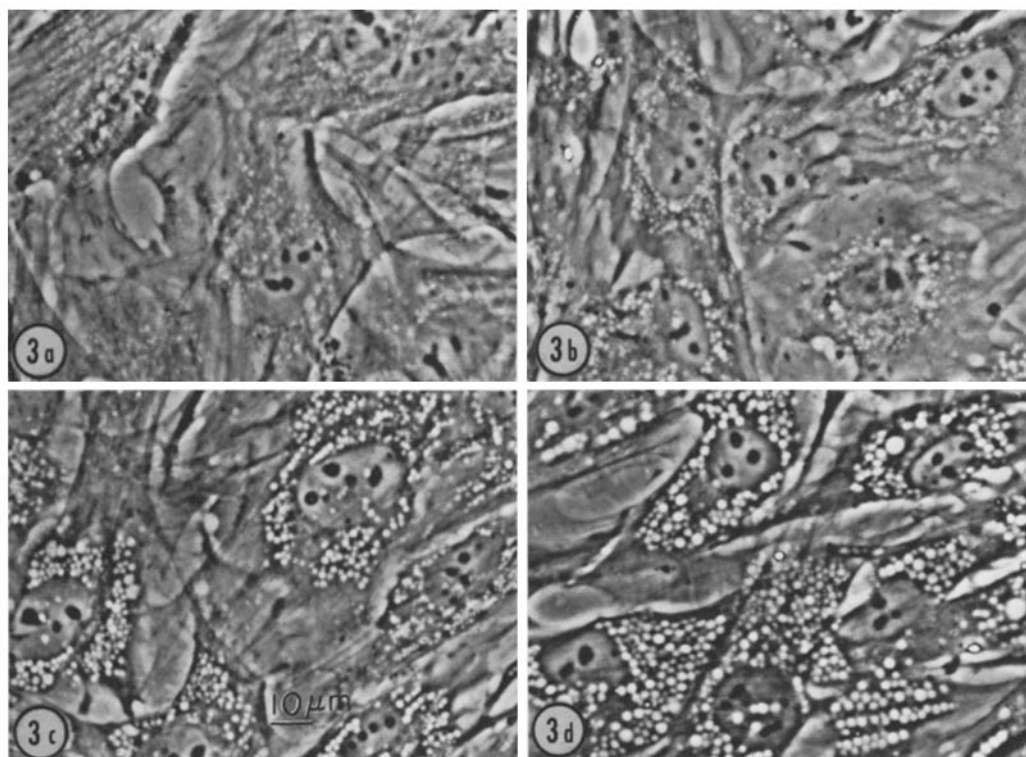

FIGURE 3 Phase-contrast microscopy of chloroquine-treated fibroblasts. The times of exposure to 100 μM chloroquine were: 20 min (a), 40 min (b), 85 min (c), and 180 min (d). $\times$ 550.
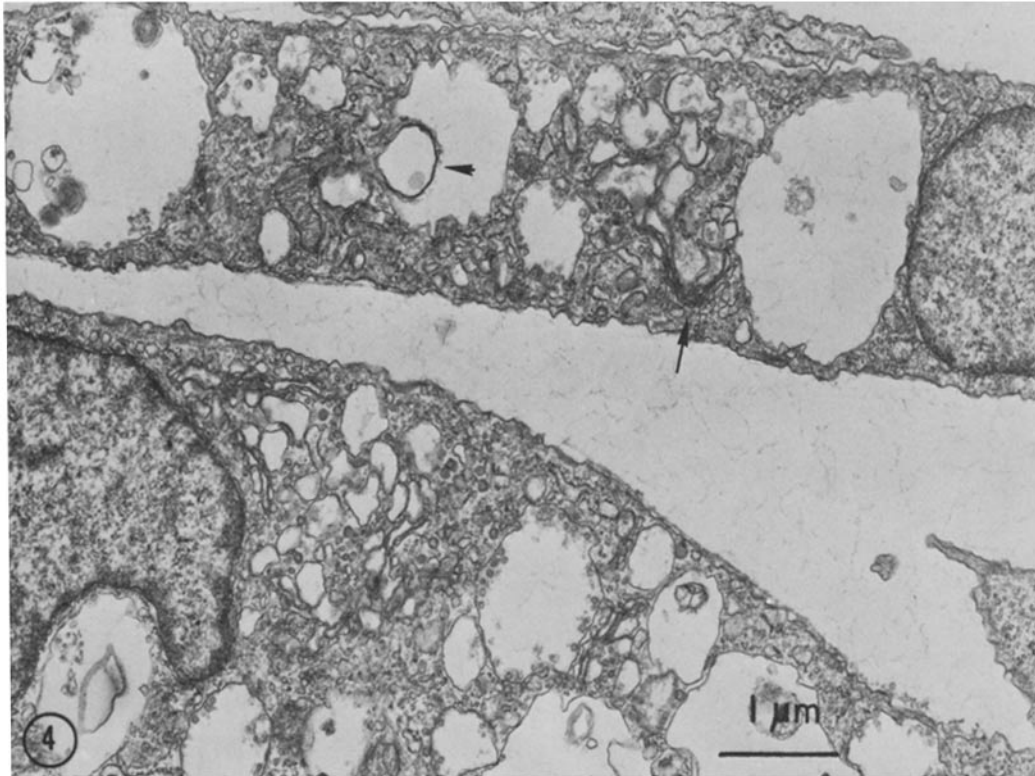
FIGURE 4   Electron microscopy of cells treated with 100 μM chloroquine for 140 min. Numerous electron-lucent vacuoles are present. The largest ones fill the whole thickness of the cytoplasm. Some vacuoles seem to arise from dilated parts of the Golgi complex (arrow). The arrowhead indicates an invagination of the membrane of a vacuole, which could lead to the entrapment of a smaller vacuole. × 15,500.

drug, only a small part of the chloroquine left the cells during the subsequent 2 h.

Fig. 7 shows the effect of the pH of the medium on the uptake of chloroquine, expressed by the concentration ratio cells/medium. This ratio was calculated assuming 3.5 μl of cell water per mg protein for fibroblasts exposed 20 min to chloroquine, and 5 μl/mg protein for cells after 200-min exposure. Morphological observations indeed suggest an important increase in cell volume resulting from the development of the vacuoles. The medium concentrations were corrected for the uptake by cells. This is important when the initial chloroquine concentration in the medium is 10 μM. After 200-min exposure at pH 7.4, the medium concentration falls to about 7 μM.

There is an obvious pH dependence of chloroquine uptake in cells exposed to the low concentration of the drug. The concentration ratio is multi-plied by 3-4 from pH 6.6 to pH 7.4. In cells exposed to 100 μM chloroquine the differences are less pronounced and, after 200 min, there may even be a decrease in the concentration ratio at high pH.

Subcellular Fractionation of Chloroquine-Treated Cells

Postnuclear supernates prepared from fibroblasts exposed to 100 μM chloroquine for 2 h and from control fibroblasts were analyzed by isopycnic density gradient centrifugation (Fig. 8). After chloroquine treatment, acid phosphatase and N-acetyl-β-glucosaminidase, two marker enzymes for lysosomes, equilibrated at lower densities than they did in the case of control cells. The density distribution of cytochrome oxidase, a marker enzyme for mitochondria, was unaffected by the
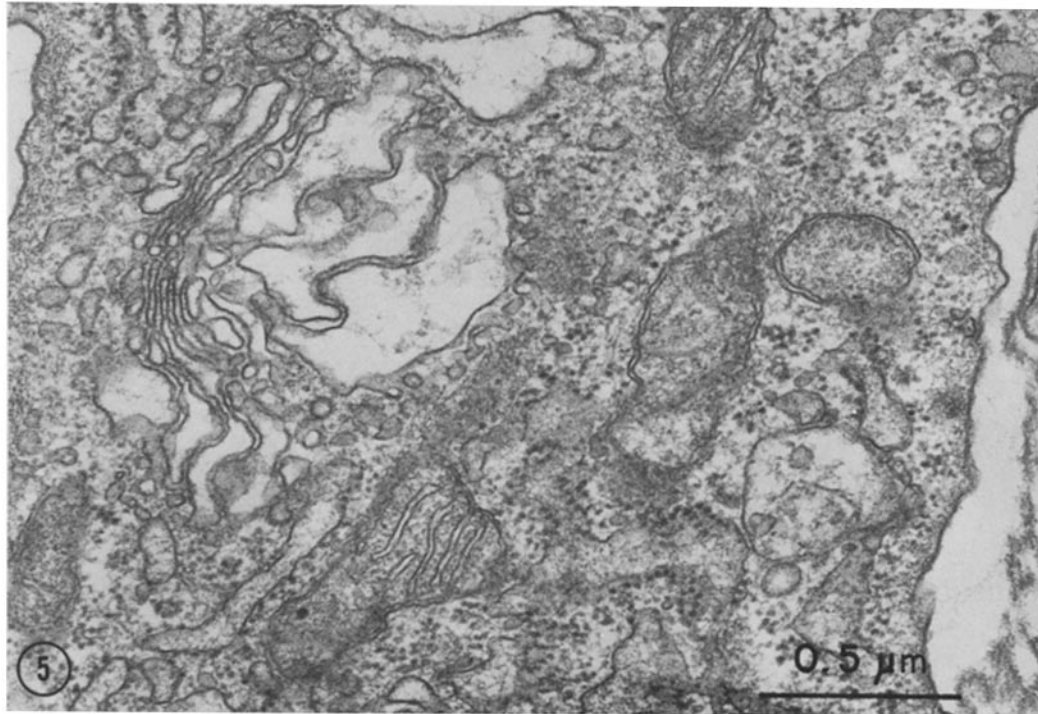
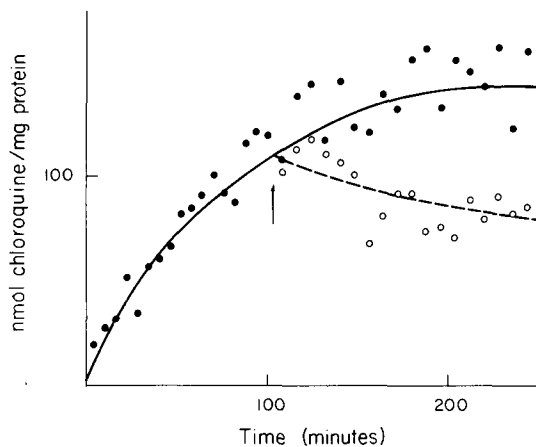FIGURE 5   Dilated Golgi complex in cells fixed after 30-min exposure to 100 μM chloroquine. × 54,000.

FIGURE 6   The uptake of chloroquine into cells from medium containing 100 μM chloroquine. At the time indicated by the arrow, medium in some tubes was changed to control medium (open symbols).

treatment, as was that of catalase. The slight shift of 5'-nucleotidase activity (plasma membranes and related structures) to higher density was greater in this particular experiment than it was in most, but it appears to be reproducible. The distribution of

5'-nucleotidase followed closely that of the lysosomal enzymes in chloroquine-treated cells. The vast bulk of the chloroquine entered the gradient and equilibrated with a broad density distribution, clearly different from that of cytochrome oxidase and catalase. The peak of chloroquine occurred at a somewhat lower density (1.12–1.13) than did the peaks of all other constituents.

The Effect of Chloroquine on Proteolysis In Vitro

Lysosomes, isolated from rat liver by the method of Leighton et al. (14), were mixed with acid-denatured bovine globin at pH 4.4 in acetate buffer as described by Coffey and de Duve (5). The increase with time of ninhydrin-reactive material was measured in the presence and absence of 100 mM chloroquine diphosphate. This high concentration was used because it seemed likely that the concentration of chloroquine within the lysosomes is high (see Discussion). The results of this experiment are shown in Fig. 9. The drug did have some inhibitory effect on proteolysis. In this experiment we followed the increase in ninhydrin reactivity, a
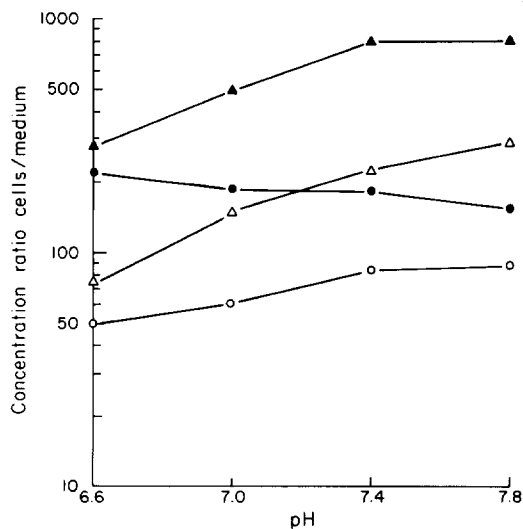
FIGURE 7 The effect of the pH of the medium on the uptake of chloroquine. The medium was buffered with 7.5 mM tricine. The concentration ratio was calculated as described in the text. Filled symbols, 200-min exposure; open symbols, 20-min exposure; circles, 100 μM chloroquine; triangles, 10 μM chloroquine.
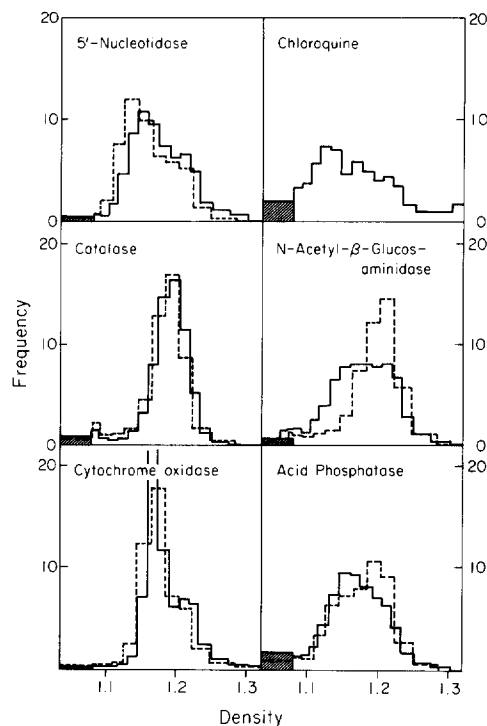

FIGURE 8 Density distributions obtained after isopycnic centrifugation of postnuclear supernates prepared from control cells (dotted line) and from cells exposed for 2 h to 100 μM chloroquine (solid line). Histograms were constructed as described by Leighton et al. (14). Percent recoveries for control and chloroquine-treated cells: cytochrome oxidase, 73.8 and 77.3; acid phosphatase, 99.5 and 92.4; catalase, 88.5 and 86.2; N-acetyl-β-glucosaminidase, 81.5 and 84.6; 5'-nucleotidase, 121.7 and 138.2; chloroquine, 100.3.

measure of the number of peptide bonds broken, while in the experiments with fibroblasts we measured the appearance of trichloroacetic acid-soluble protein fragments. Initial proteolytic attack on globin requires the action of cathepsin D. In in vitro experiments we have been unable to detect any effect of chloroquine on this enzyme, or on the activity of cathepsins A and C.

In confirmation of a previous report (6), we have found a partial inhibition by chloroquine of cathepsin B. The component of this group of enzymes that hydrolyzed benzoylarginylnaphthylamide, usually designated cathepsin B_1, was completely inhibited by high enough concentrations of chloroquine. The concentration dependence of this inhibition is shown in Fig. 10. Half maximal inhibition occurs at a concentration of about 5 mM.

DISCUSSION

The vacuoles that develop in our chloroquine-treated fibroblasts are very similar to those described by Fedorko et al. (9) in macrophages and L cells exposed to the same drug. The authors noticed that the vacuoles develop in the Golgi

region and that pinosomes and lysosomal granules frequently fuse with them. They concluded that chloroquine acts initially on Golgi vesicles (primary lysosomes?) or smooth endoplasmic reticulum vesicles, and on lysosomal granules. The images of peripheral dilatation of the Golgi complex that we observed (Figs. 4 and 5) also point to a possible role of the Golgi apparatus, or of the closely associated smooth endoplasmic reticulum, in the development of the vacuoles.

Using fluorescence microscopy and histochemical staining Fedorko et al. (9) demonstrated that the "toxic" vacuoles contain chloroquine and acid phosphatase, in confirmation of early observations by Allison and Young (1) that chloroquine is
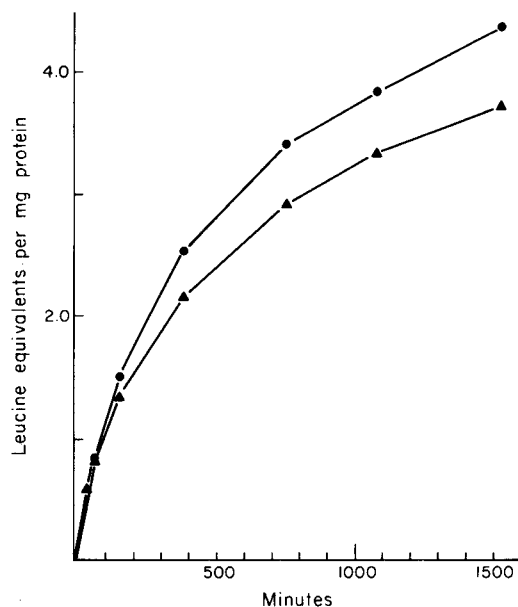
FIGURE 9 Release of ninhydrin-reactive material from bovine globin incubated with rat liver lysosomes in 0.1 M acetate buffer, pH 4.4, in the presence (▲) and absence (●) of 0.1 M chloroquine.

concentrated within lysosomes. Our fractionation experiments (Fig. 8) indicate that the bulk of the chloroquine taken up by fibroblasts is particle-bound and that its density distribution is compatible with the lysosomal nature of the vacuoles. The density distribution of lysosomes is clearly affected by the chloroquine treatment and it will be shown below that the shift of the lysosomal enzymes towards lower densities is explainable on the basis of a transformation of lysosomes into swollen vacuoles, filled with a chloroquine solution. The staining by neutral red of the vacuoles also suggests that they are lysosomes. However, on the basis of the results of Fig. 8, we cannot exclude the possibility that part of the chloroquine is associated with other structures, such as low density Golgi elements, or plasma membranes and related structures.

The lysosomal pH (8, 13) can account for an extensive accumulation of a weak base such as chloroquine (12) inside these particles. The pH difference between lysosomes (pH 4) and the culture medium (pH 7) would result in a 1,000-fold concentration within the particles, provided only the uncharged species of chloroquine, but not its
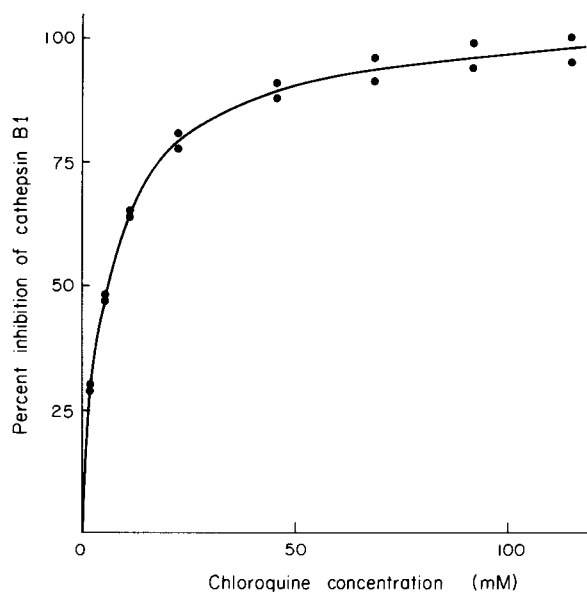

FIGURE 10 Inhibition of cathepsin B_1 by chloroquine. The enzyme preparation was a postnuclear supernate from a rat liver homogenate.

protonated species,[1] were able to pass freely across the cell and lysosome membranes (7). Indeed, the concentration ratio cells/pH 7 medium was estimated at 500 after 200-min exposure to 10 μM chloroquine (Fig. 7). The ratio lysosomes/medium could be at least four times higher. Moreover, except for cells exposed 200 min to 100 μM chloroquine, the concentration in cells increases with the pH of the medium, as would be expected. However, this augmentation is at the most fourfold from pH 6.6 to 7.6, whereas a change of one pH unit should theoretically result in a 10-fold change in the concentration ratio lysosomes/medium. It is probable that, beyond certain limits, the active processes required for extensive accumulation of chloroquine to occur are overwhelmed. The uptake of large amounts of the drug implies not only an important production of membrane for the vacuoles, but also the secretion of protons into them. Indeed, at pH 4, the concentration of free protons is only 100 μM. Hence many more protons have to be generated within the lysosomes to replace those lost during the protonation of the chloroquine molecules. We have no idea what the buffering capacity of the lysosomal content is, but it seems unlikely to be high enough to sustain a low pH in the presence of large amounts of chloroquine. Consequently a proton pump is required to allow accumulation of chloroquine within the lysosomes. The inhibition of these energy-requiring processes is the most probable explanation for the reduction of chloroquine uptake in the cold and in cells exposed to metabolic inhibitors (17). We observed that 10 mM NaF impedes the development of the chloroquine vacuoles.

From the data of Figs. 6 and 7 we may estimate the final chloroquine concentration in the whole cells at about 25 mM when the initial medium concentration is 100 μM. The concentration in the lysosomes could be three to four times higher, i.e. 75-100 mM. In cells exposed to 10 μM chloroquine at pH 7.4, the final concentration of the drug is only about 5 mM, but it is probable that the lysosome volume is distinctly smaller than 25% of

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[1] Chloroquine has two basic groups, with pKs of 8.1 and 10.1 (12). For concentration to occur, it suffices that the membranes be impermeable to the doubly protonated form. Impermeability to the monoprotonated form is probable, since monovalent bases such as neutral red are also concentrated in lysosomes.

the cell volume. Consequently the concentration of chloroquine in the lysosomes may be close to 50 mM.

Since the chloroquine is doubly charged inside lysosomes, it will carry with it two counterions, probably chloride, that will triple the osmotic potential. A solution in which anions such as chloride contribute two-thirds of the osmolarity must have a comparatively low density. Hence it is not surprising that the chloroquine-containing, swollen lysosomes, which remain in osmotic equilibrium with the sucrose solution outside the particles, equilibrate at lower densities in a sucrose gradient. Moreover, those lysosomes that contain the most chloroquine would be expected to have the lowest density. This is probably the reason why the peak of the chloroquine distribution occurred at densities of 1.12-1.13, as compared with 1.15-1.16 for the lysosomal enzymes (Fig. 8).

We know for certain that extracellular proteins taken up by endocytosis are digested inside lysosomes. Chloroquine has been reported to inhibit the digestion of hemoglobin within secondary lysosomes of plasmodia (10). We have shown that cellular protein breakdown is inhibited in chloroquine-treated fibroblasts. Since this drug accumulates inside lysosomes, and modifies their morphological and physical properties, it is plausible that some essential step of cellular protein breakdown, common to proteins of both slow and rapid turnover, is localized in the lysosomes. Obviously, this step could be the degradation of sequestered cellular proteins to their constituent amino acids by the lysosomal proteases. It is, of course, impossible to exclude the possibility that the low concentration of chloroquine in other parts of the cell may interfere with cellular protein breakdown in some way.

The simplest explanation for the inhibition of cellular protein catabolism by chloroquine would be the inhibition of proteolytic enzymes in the lysosomes. We have found that cathepsin D is not affected by chloroquine. This enzyme was considered previously as the main lysosomal protease, since the digestion of hemoglobin by extracts of rat liver lysosomes is largely the consequence of its action (5). However, recent work has shown that this enzyme has little or no activity on a number of other proteins (11). On the other hand, cathepsin B_1 acts on a rather wide assortment of proteins (3, 4), and this enzyme is probably responsible for the

primary proteolytic attack on many proteins within lysosomes. Cathepsin B_1 is inhibited severely by chloroquine at a concentration that must be present within the lysosomes of chloroquine-treated cells. This inhibition could thus account for the effect of chloroquine on intracellular protein breakdown.

In view of the absence of effect of chloroquine on cathepsin D, the inhibition of hemoglobin digestion in the lysosomes of plasmodia is somewhat puzzling. However, the proteases in plasmodia may have properties different from those of rat liver. Homewood et al. (10) have suggested that chloroquine may neutralize the acidity within the lysosomes of plasmodia, raising the intralysosomal pH to a point where acid hydrolases no longer act. However, as mentioned above, very little chloroquine would be required for this neutralization and, unless the pH within the lysosomes remained low, there would be no accumulation of the drug within lysosomes.

Recently, Lie and Schofield (15) showed that intralysosomal digestion of mucopolysaccharides is impaired in fibroblasts exposed to 10–20 μM chloroquine. Thus, in addition to cathepsin B_1, other lysosomal enzymes could be inhibited by chloroquine.

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