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THE EFFECTS OF AMMONIUM IONS AND CHLOROQUINE ON UPTAKE AND DEGRADATION OF ^{125}I-LABELED ASIALO-FETUIN IN ISOLATED RAT HEPATOCYTES

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Abstract—The effects of NH_4^+ and chloroquine on the uptake, the degradation and the intracellular distribution of ^{125}I-labeled asialo-fetuin in isolated rat hepatocytes were investigated. Both ammonium ions and chloroquine inhibited asialo-fetuin degradation; chloroquine in high concentrations reduced uptake as well. Both compounds reduced the equilibrium density of the lysosomes in sucrose gradients. Labeled asialo-fetuin did not seem to accumulate in the lysosomes of cells exposed to NH_4^+ or chloroquine. The possibility that both compounds retard the fusion between lysosomes and phagosomes is discussed.

We have previously shown that desialylated fetuin, labelled with ^{125}I, is taken up *in vitro* in isolated rat hepatocytes by a process involving high-affinity receptors on the hepatocyte plasma membrane [1–4]. Asialo-fetuin was degraded in the hepatocytes, as indicated by the release of acid-soluble radioactivity from the cells. The release started about 15 min after the addition of labelled protein to the cells, and the rate of degradation was subsequently constant for at least 60 min [1–4].

Degradation occurs in the lysosomes. Degradation products rapidly escape from the lysosomes to the cytoplasm and subsequently to the medium [1, 5].

The lag period of 15 min between the start of the uptake and the onset of the degradation points to the involvement of mechanisms for transport of the endocytosed protein through the cytoplasm and/or a mechanism for achieving fusion between endocytic vacuoles and lysosomes. It has been shown that colchicine (an inhibitor of microtubular function) as well as cytochalasin B (an inhibitor of microfilaments) inhibit degradation, presumably by interfering with intracellular transport of asialo-fetuin [6].

In the present investigation, we have studied the effects of two weak bases, chloroquine and ammonia, on the intracellular distribution of labelled asialo-fetuin in order to obtain additional information about the intracellular processing of asialo-fetuin. Being weak bases, both chloroquine [7] and ammonia [9] accumulate in the lysosomes. They penetrate the lysosomal membrane in their uncharged form and accumulate in the lysosomes as a consequence of protonation [8]. This accumulation of osmotically active particles leads to swelling of the lysosomes and to an inhibition of the intralysosomal proteolysis [7, 8]. In addition, chloroquine inhibits the lysosomal protease cathepsin B [8]. One possible effect of the two bases would therefore be the accumulation of undegraded asialo-fetuin in the lysosomes, making the labelled protein detectable in these organelles sooner than in control cells.

However, it is not unlikely that chloroquine and ammonia have other effects on the intracellular processing of endocytosed asialo-fetuin. Other possible points of action are on the binding of asialo-fetuin to the plasma membrane, the formation of endocytic vesicles, the transport of these vesicles through the cytoplasm, and the fusion between endocytic vesicles and lysosomes. In the present investigation, we have tried to distinguish between these possibilities by studying the rate of uptake and the intracellular distribution patterns of asialo-fetuin in the presence and absence of chloroquine and ammonium ions. Some of the results presented here have been published in preliminary reports [3, 4].

METHODS AND MATERIALS

Chemicals. ^{125}I-labelled asialo-fetuin was prepared as described previously [2]. The specific activity of the labelled protein was about 26×10^6 c.p.m. per nmole. Collagenase (type I), bovine serum albumin (fraction V) and enzyme substrates were from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin was purified by charcoal treatment before use [10]. Dibutyl phthalate and dinonyl phthalate were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K.

Preparation of purified rat hepatocytes. Isolated rat liver cells were prepared from male Wistar rats, 200–250 g, by the collagenase perfusion method [11], as described previously [12, 13]. Hepatocytes were separated from nonparenchymal liver cells by low-speed centrifugation [13].

Cell incubations. Purified hepatocytes were suspended in incubation medium [1] and incubated with the desired additions in 10–50 ml Erlenmeyer flasks in a shaking water bath at 37°. The cell concentration was 5×10^6 cells/ml. To determine the amount of cell-associated ^{125}I-labelled asialo-fetuin, the cells were separated from the medium by centrifugation for 1 min through 250 μl of dibutyl phthalate: 250 μl samples of cell suspension were layered on top of the oil in 750 μl polypropylene centrifuge tubes and the tubes centrifuged for 30 sec at 7000 r.p.m. in a microfuge [1, 14]. Degradation of ^{125}I-labelled asialo-

fetuin was followed either by measuring acid-soluble radioactivity released to the medium (and remaining on top of the oil) from the cells, or, alternatively, by assessing the increase in total acid-soluble radioactivity in the cell suspension. Cold phosphotungstic acid (final concentration 2%, w/v) was used for separating acid soluble and acid precipitable radioactive compounds.

Homogenization and subcellular fractionation of hepatocytes. In order to study the intracellular distribution of asialo-fetuin, the cells were first incubated for 10 min in the presence of about 30 nM asialo-fetuin. Then the suspension was placed on ice, the cells were centrifuged down, and the medium was replaced with fresh medium containing no labelled protein. Ammonium chloride (10 mM) or chloroquine (0.1 mM) was added to a portion of the cell suspension. The incubation was then continued at 37°.

Samples of the cell suspension, usually containing 50×10^6 cells, were removed at suitable time points. The cells were separated from the medium by centrifugation for 30 sec at 40 *g* and washed three times in ice-cold 0.25 M sucrose solution. The cells were suspended in ice-cold 0.25 M sucrose (final volume: 5 ml) and homogenized in a chilled Dounce homogenizer with a tight-fitting pestle [1, 2].

Subcellular fractionation of the homogenate was done either by differential centrifugation [15] or by isopycnic centrifugation. In the latter case 4 ml of postnuclear fraction from 20×10^6 cells were layered on top of linear sucrose gradients with densities ranging from 1.10 to 1.27. The volume of the gradient itself was 34 ml. The gradients were prepared in tubes fitting the Beckman SW-27 rotor and the tubes were centrifuged at 2° for 4 hr at 25,000 r.p.m. After the centrifugation the gradients were pushed out of the tubes by introducing heavy sucrose at the bottom of the tubes. Two millilitre fractions were collected.

Radioactivities were determined in a Packard auto gamma spectrometer (Packard Instrument Co. Inc., Downers Grove, IL, U.S.A).

Biochemical determinations. β-acetylglucosaminidase (EC 3.2.1.30), acid phosphatase (EC 3.1.3.2) and cathepsin B (EC 3.4.22.1) were determined as described by Barrett [16]. 5'nucleotidase (EC 3.1.3.5) was assayed according to El-Aaser and Reid [17] and protein according to Lowry *et al.* [18].

RESULTS

Effects of ammonium ions and chloroquine on the rates of uptake and degradation of ^{125}I-labelled asialo-fetuin. The effects of NH_4^+ (10 mM) and chloroquine (0.1 mM) on the uptake and the degradation of ^{125}I-labelled asialo-fetuin in rat hepatocytes are shown in Fig. 1. The initial concentration of asialo-fetuin in experiments concerning uptake was about 30 nM. At this concentration the rate of asialo-fetuin uptake was approximately half-maximal in control cells [1].

With the concentrations of NH_4^+ and chloroquine used, neither of these compounds had any measurable effects on the initial rate of uptake of asialo-fetuin by the hepatocytes (Fig. 1A). Both compounds inhibited markedly, however, the rate of formation of acid-soluble radioactivity from the cells. Figure 1B shows that control cells degraded about twice as much labelled asialo-fetuin during the first hour of incubation (in asialo-fetuin-free medium) as cells incubated in presence of either NH_4^+ (10 mM) or chloroquine (0.1 mM).

Ammonium ions in concentrations up to 10 mM did not affect the rate of uptake of asialo-fetuin by the hepatocytes. Chloroquine, on the other hand, reduced the uptake of asialo-fetuin by the cells when its concentration exceeded 0.1 mM. At concentrations above 1 mM, chloroquine almost completely inhibited both uptake and degradation of asialo-fetuin by the cells (Fig. 2). We were not able to homogenize properly hepatocytes that had been

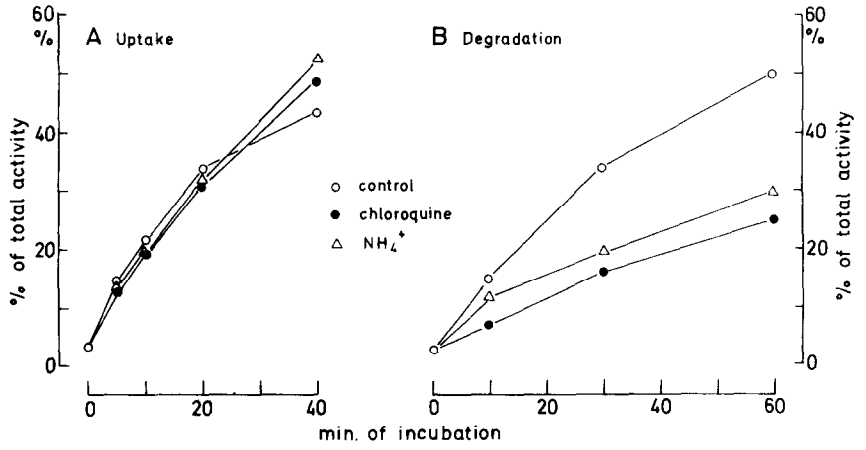

Fig. 1. The effects of NH^+ (10 mM) and chloroquine (0.1 mM) on the uptake (A) and the degradation (B) of ^{125}I-labelled asialo-fetuin. Panel A: Hepatocytes (5×10^6 cells/ml) were incubated with 30 nM labelled asialo-fetuin in presence of NH_4^+ (▲), chloroquine (●) or with no additions (○). Panel B: Hepatocytes were preincubated for 10 min with labelled asialo-fetuin. The cells were then washed free of medium and reincubated without labelled asialo-fetuin, but with ammonium or chloroquine. Symbols are as defined in A. The incubations were carried out at 37°. Uptake is measured as cell-associated radioactivity [1]. Both uptake and degradation are presented as per cent of total radioactivity.

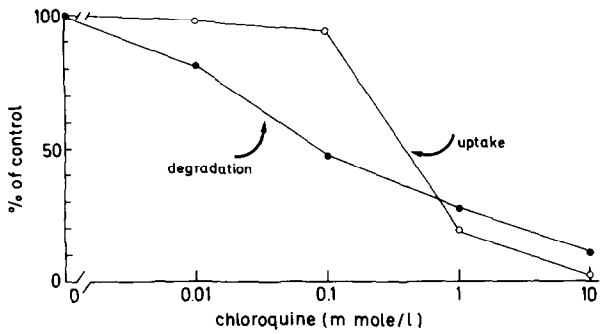

Fig. 2. The effect of chloroquine on the uptake (○) and the degradation (●) of ^{125}I-labelled asialo-fetuin. Uptake was measured as net increase in radioactivity in cells that had been incubated for 10 min with labelled asialo-fetuin. The cells were exposed to 0, 0.01, 0.1, 1 and 10 mM chloroquine and the uptake is expressed as per cent of that measured in the control cells. Degradation of asialo-fetuin was measured in cells that had been preincubated for 10 min with 30 nM ^{125}I-asialo-fetuin and then reincubated for 30 min in fresh medium without labelled protein but with different concentrations of chloroquine. The increase in acid-soluble radioactivity was measured, and degradation is expressed as per cent of control.

exposed to chloroquine in concentrations above 1 mM. Chloroquine evidently rendered the plasma membrane very resistant to mechanical stress. In order to obtain a cell-free homogenate, the tissue grinding had to be performed for such a long time that the cell organelles were severely damaged. Homogenization problems were avoided when the chloroquine concentrations were kept at or below 0.1 mM and such concentrations of chloroquine were chosen in subsequent experiments where cells were fractionated following the exposure to chloroquine.

Additive effects of chloroquine and colchicine or ammonia and colchicine. In order to obtain information about the effect of chloroquine and ammonia on the intracellular transport of asialo-fetuin, experiments were performed to determine the combined effect on degradation of either of the two bases together with colchicine. Cells which had been incubated with ^{125}I-asialo-fetuin and then washed were exposed to chloroquine and/or colchicine in one experiment, and ammonium chloride and/or colchicine in another experiment. The results are summarized in Table 1. As colchicine takes at least 20 min to exert its full effect in this system [6], only values obtained 40 min or more after washing of the cells are tabulated. It may be concluded from Table 1 that the combined effect (degradation measured relative to untreated cells) of either of the two bases plus colchicine is close to the product of the effect of the base multiplied by the effect of colchicine. This result is consistent with the idea that colchicine acts on a stage of the intracellular processing which is different from the stage which is affected by the bases chloroquine or ammonia.

Differential centrifugation. The distribution in fractions obtained by differential centrifugation of the plasma membrane marker enzyme 5′nucleotidase was relatively unaffected by the time of incubation and also by the presence of ammonium chloride or chloroquine in the incubation medium. This enzyme was located mainly in the microsomal fraction (Fig. 3A). The distribution pattern of 5′nucleotidase was quite similar to that of the radioactivity early after the start of the uptake of labelled asialo-fetuin. Later in the incubation, the distribution pattern of radioactivity changed, coming to resemble the distribution pattern for lysosomal marker enzymes (Fig. 3B) in the control cells.

Chloroquine markedly affected the distribution of lysosomal enzymes, causing a shift towards *lighter* fractions: as the incubation progressed, more and more of the lysosomal enzymes was found in the microsomal and the soluble fractions, while the enzyme activities (also the relative specific activities) in the nuclear, mitochondrial and light mitochondrial fractions decreased (Fig. 3B).

Ammonium ions had an effect on the distribution pattern of the lysosomal marker enzymes which resembled that of chloroquine. The lysosomal

Table 1. Additive effects of colchicine and either chloroquine or ammonia*

	Degradation (fraction of degradation in untreated cells)			
Compound(s) added	I Chloroquine	II Colchicine	III Chloroquine + colchicine	Product of values in cols. I and II
Time (min)				
40	0.591	0.757	0.477	0.447
60	0.644	0.687	0.460	0.442
90	0.674	0.667	0.450	0.450
Compound(s) added	Ammonium chloride	Colchicine	Ammonium chloride + colchicine	
Time (min)				
40	0.561	0.774	0.454	0.434
60	0.489	0.691	0.363	0.338
90	0.435	0.671	0.342	0.292

* Concentrations used were: chloroquine 0.05 mM, ammonium chloride 5 mM, colchicine 0.1 mM. Cells were exposed to 15 nM labelled asialo-fetuin for 15 min and washed before the compounds were added. Time points indicated refer to time elapsed after addition of the compounds.

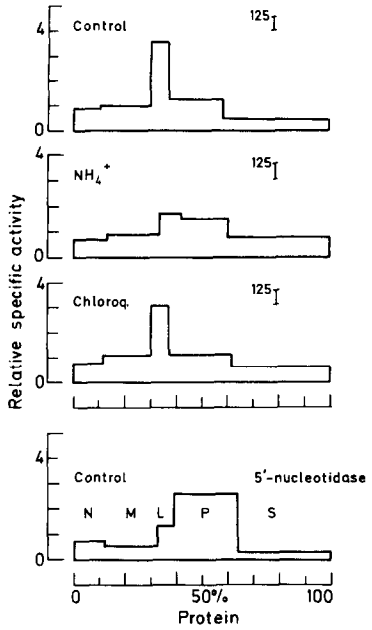

Fig. 3A. The three upper curves show the distribution of radioactivity in fractions prepared by differential centrifugation of homogenates from control cells, or cells that had been treated for 60 min with chloroquine (0.1 mM) or NH_4^+ (10 mM) following a 10 min preincubation with labelled asialo-fetuin. The lower histogram shows the distribution of 5'nucleotidase for control cells. NH_4^+ or chloroquine did not affect the distribution of this enzyme significantly. Radioactivities or enzyme activity are presented as relative specific activities of fractions: percentage of total recovered activity/percentage of total protein. Abscissa: relative protein content of fractions, cumulatively from left to right. The total recovered activity of 5'nucleotidase varied between 99 and 108 per cent of that in the original homogenate. The recovery of protein and radioactivity was between 89 and 97 per cent. Five experiments were performed. The results obtained were closely similar in all the experiments.

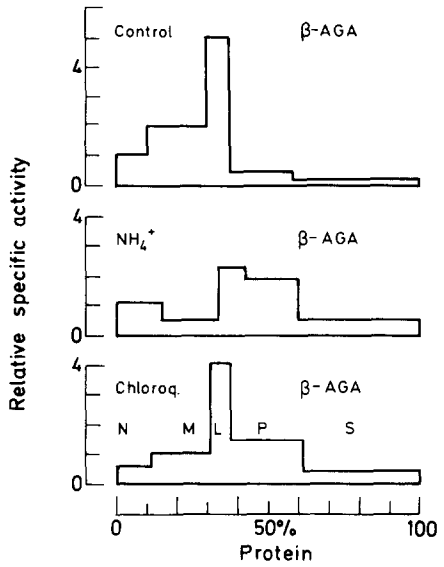

Fig. 3B. Distribution of β-acctylglucosaminidase in fractions prepared by differential centrifugation of homogenates from hepatocytes treated as described in the legend to Fig. 3A. The total recovered activity of the enzyme varied between 86 and 97 per cent of that in the homogenate. Five experiments were done. N: nuclear fraction, M: heavy mitochondrial fraction, L: light mitochondrial fraction, P: particulate fraction (or microsomal fraction), S: final supernatant.

enzyme activities increased several-fold in the microsomal fraction, at the expense of the activities in the two mitochondrial fractions (Fig. 3B). Ammonium ions also changed the distribution of radioactivity towards lighter fractions, as if ammonium ions rendered subcellular particles containing radioactivity less dense (or smaller) (Fig. 3A).

Fractionation of control cells by differential centrifugation showed, as described previously [1–4] that the radioactivity was located initially mainly in the microsomal (P) fraction. As the incubation progressed, there was a gradual increase in radioactivity in the heavier fractions (the mitochondrial fractions and the nuclear fractions). The distribution pattern of radioactivity for cells that had been incubated for more than one hour became similar to that of the lysosomal enzymes (Fig. 3B). This similarity is partly incidental, as other evidence indicates that most of the ingested asialo-fetuin is not located in the lysosomes (see Ref. 2 and the next section).

The addition of chloroquine to cells that had been preincubated with labelled asialo-fetuin led to very small changes in the subcellular distribution pattern of radioactivity, compared to control cells (Fig. 3A). At any given time-point, cells incubated with chloroquine contained more radioactivity than control cells, due to the inhibition of degradation of labelled protein by chloroquine. The additional radioactivity in chloroquine-treated cells was, however, distributed among the subcellular fractions in such a way that the relative specific activities were nearly unaffected.

The salient points of the experiments with differential centrifugation are the following: (1) in control cells, the distribution of radioactivity changed towards heavier fractions with increasing time of incubation, while the distributions of the marker enzymes were little changed; (2) in chloroquine-treated cells, the distribution of radioactivity was nearly the same as in control cells, while the lysosomal marker enzymes were shifted towards lighter fractions; (3) ammonium ions also changed the distribution of lysosomal marker enzymes towards lighter fractions; coincidentally, there was also a small shift of the distribution of radioactivity in the same direction. In Fig. 3B the activity of only β-acetylglucosaminidase is shown. However, the activities of cathepsin B and acid phosphatase gave closely similar results.

Results obtained with isopycnic centrifugation. Homogenates from cells exposed for 10 min to asialofetuin and then incubated in medium without labelled protein were also fractionated by density gradient centrifugation. The results of such experiments are shown in Figs. 4 and 5. The distribution curves for radioactivity in the gradients were rather similar for control cells and cells treated with chloro-

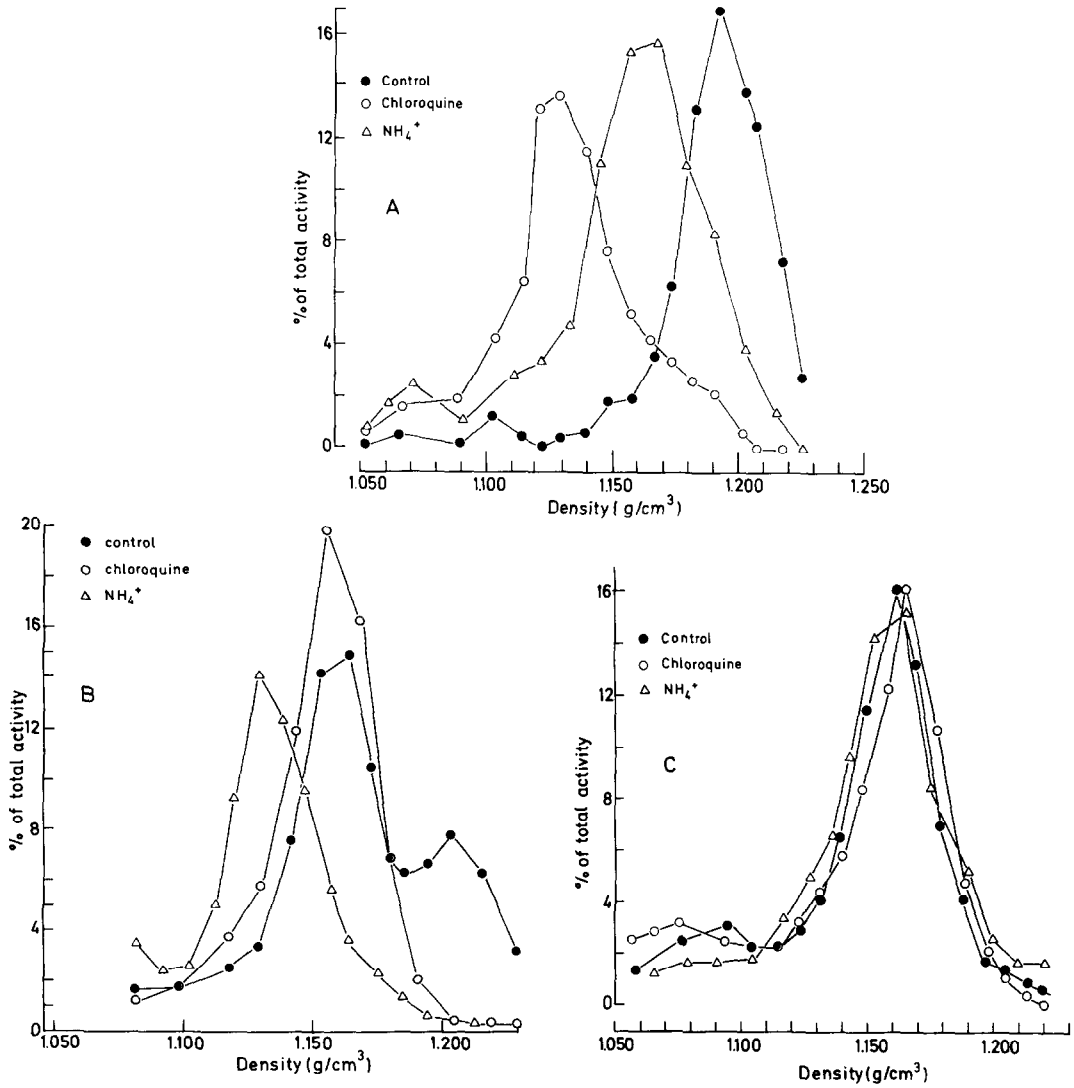

Fig. 4. Distributions histograms of enzyme activities and radioactivity in sucrose gradients. Hepatocytes were incubated for 10 min with 30 nM labelled asialo-fetuin, then washed and reincubated in fresh medium with 10 mM NH_4^+ (▲), 0.1 mM chloroquine (○) or with no additions (●). One hour after the start of the reincubation, the cells were separated from the medium, homogenized in cold 0.25 M sucrose and fractionated by isopycnic centrifugation as described in the method section. ^{125}I-radioactivity (B), β-acetylglucosaminidase (A) or 5′nucleotidase (C) were measured in the subcellular fractions. The enzyme activity or radioactivity in the fractions are presented as per cent of total recovered activity in the gradient and is plotted against the density. The recovery of the components measured in the gradients as per cent of that layered above the gradient initially was in the experiment shown here between 85 and 105 per cent. Seven different experiments were performed. The results obtained were closely similar in the different experiments.

quine, provided the cells were incubated for less than 60 min. Most of the radioactivity that had entered the gradient was found in a band at an average density between 1.12 (at the start of the incubation) and 1.16 (after 60 min). We have discussed elsewhere the possibility that this major radioactivity peak represents endocytic vesicles [2]. For control cells incubated for more than one hour, a second peak of radioactivity appeared at a density of about 1.20 (Fig. 4B). This peak coincided, as pointed out earlier [2–4], with that of the lysosomal marker enzymes and probably represented labelled asialo-fetuin accumulated within these organelles [2, 5]. The distribution curves for radioactivity shown in Fig. 4B indicate that NH_4^+ changes the equilibrium density of the organelles containing radioactivity to lower values than those seen for chloroquine-treated cells and control cells. This finding is in agreement with what was seen in experiments where cells were fractionated by differential centrifugation. In that case too, NH_4^+ led to a change in the distribution of radioactivity towards lighter fractions.

The distribution of 5′nucleotidase in fractions obtained by density gradient centrifugation resembled that of the radioactivity (Fig. 4C), which could mean that the bulk of radioactivity in the cells was on the plasma membrane. This is, however, as pointed out elsewhere [2], very unlikely, as very little

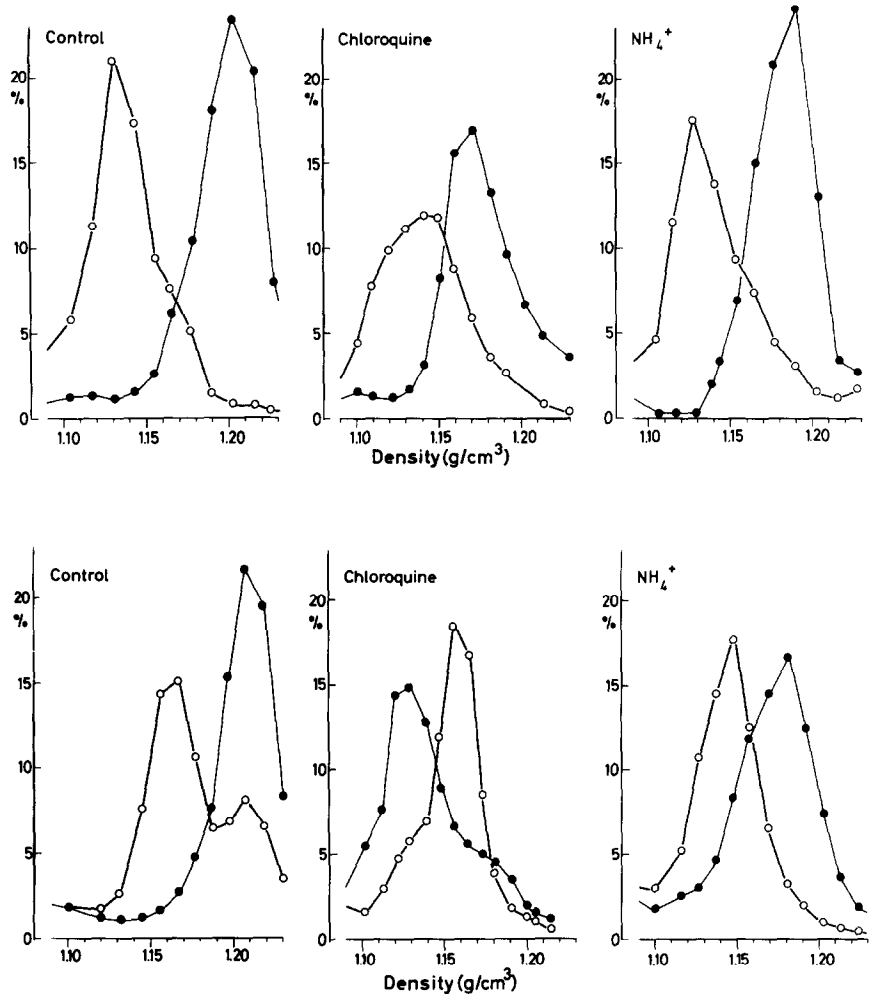

Fig. 5. Effects of NH_4^+ (curves to the right) and chloroquine (curves in the middle) on the distribution patterns of β-acetylglucosaminidase (●) and radioactivity (○) after centrifuging in sucrose gradients. Hepatocytes were first preincubated for 10 min with 10 nM ^{125}I-asialo-fetuin. The cells were then washed and reincubated in fresh medium. Chloroquine (0.1 mM) or NH_4Cl (10 mM) were added to portions of the washed cells. Control cells as well as cells that had been exposed to NH_4^+ and chloroquine were removed from the incubator after 20 min (upper panel) and 90 min (lower panel), homogenized and fractionated as described in the method section. The data are presented as described in the legend to Fig. 4.

labelled protein is actually bound to the plasma membrane when the cells are homogenized [2]. In addition, the distribution of radioactivity and 5′nucleotidase was entirely different in fractions obtained by differential centrifugation, provided the cells had been incubated for some time after the uptake of asialo-fetuin (Fig. 3A). This was true both for control cells and cells incubated with chloroquine and NH_4^+. Therefore, the coincidence of the distribution curves for radioactivity and 5′nucleotidase seemed to be incidental and not the consequence of the two activities being containing in the same subcellular structure.

The distribution of lysosomal enzymes in gradients with material from chloroquine or ammonium treated cells indicated clearly that the lysosomes of these cells became lighter (Fig. 4A); after 60 min the peak activities of e.g. β-acetylglucosaminidase were found at densities of about 1.12 and 1.16 g/cm^3 in cells treated with chloroquine and ammonium ions, respectively. The corresponding value for control cells was, as pointed out previously, 1.20 g/cm^3.

Figure 5 shows the distribution patterns of a lysosomal enzyme as well as of ^{125}I-labelled asialo-fetuin after centrifugation in sucrose gradients. Again, β-acetylglucosaminidase, cathepsin B and acid phosphatase gave closely similar results. Material from hepatocytes incubated in the presence and the absence of NH_4^+ and chloroquine was used, and the histograms in Fig. 5 show the situation after the cells had been incubated for 20 min (upper panel) as well as for 90 min (lower panel). A comparison of the subcellular distribution patterns for the lysosomal enzyme and the labelled asialo-glycoprotein does not indicate that NH_4^+ or chloroquine had led to any noticeable accumulation of labelled asialo-fetuin in the lysosomes. This is true both for cells incubated for 20 min and 90 min. Cells treated with chloroquine

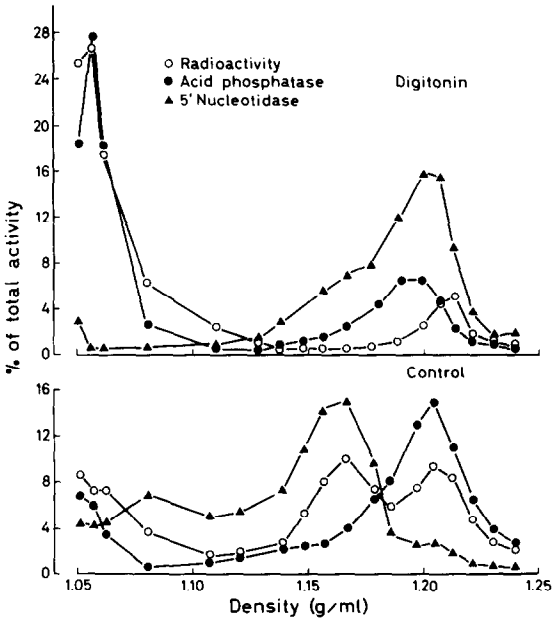

Fig. 6. Effects of digitonin on the distribution patterns of asialo-fetuin and marker enzymes in isopycnic centrifugation. The cell suspension was made 5 nM in asialo-fetuin and incubated for 90 min at 37°. Subsequent steps were precisely the same as described in the Methods section, with the following amendment: the cytoplasmic extract was divided into two equal portions of 5 ml each. To one portion was added 50 μl of a 100 mg/ml solution of digitonin, dissolved in a mixture of equal volumes of water and dimethyl sulfoxide. Portions (10 μl) were added over a period of 5 min with careful shaking between each addition, then the cytoplasmic extract was left for 10 min on ice before fractionation by isopycnic centrifuging. The lower part of the figure shows the distribution pattern for the untreated control extract, the upper part shows the distribution pattern for the portion of the extract treated with digitonin. ○, radioactivity; ●, acid phosphatase; ▲, 5'-nucleotidase.

or NH_4^+ for 90 min contain up to twice as much labelled protein as control cells. If this additional amount of labelled glycoprotein was inside lysosomes, one would expect a marked peak of radioactivity in the region of the gradient where the peak activity of the lysosomal enzyme is seen (Fig. 5).

Influence of digitonin on distributions of radioactivity and marker enzymes in sucrose gradients. In order to test the possibility that the peak of radioactivity found at $d = 1.16$ g/ml represents labelled asialo-fetuin bound to fragments of the plasma membrane, the cytoplasmic extract was treated with digitonin (1 mg/ml) before fractionation. Digitonin selectively modifies the equilibrium density of plasma membrane fragments by binding to cholesterol [19]. In Fig. 6, the distributions in the digitonin-treated homogenate are compared to the distributions in an untreated portion of the same homogenate (control). The cells had been incubated for 90 min after the addition of a small concentration of asialo-fetuin.

In the control homogenate, the distribution of radioactivity showed two peaks of similar size, one of which coincided with 5'nucleotidase at $d = 1.16$ g/ml and the other with acid phosphatase at $d = 1.20$. In the digitonin-treated homogenate, the entire peak of 5'nucleotidase activity had moved to $d = 1.19$ g/ml, but the corresponding peak of radioactivity did not move along with it. In this homogenate, there was no radioactivity peak at all at $d = 1.16$ g/ml. The main conclusion to be drawn from this experiment is that the radioactivity which appeared in the gradient at $d = 1.16$ resided in a different entity from the 5'nucleotidase activity which appeared at the same density.

DISCUSSION

The data presented here show that ammonium ions as well as chloroquine retard the release of acid-soluble radioactivity from cells that have taken up asialo-fetuin at a time when no radioactivity could be shown to be associated with lysosomes. The effects of these weak bases on the degradation of asialo-fetuin would seem to favor the possibility that early after the start of the uptake of the glycoprotein, the lysosomal degradation was so effective that no undegraded asialo-fetuin was detectable in lysosome-rich fractions.

Chloroquine in concentrations above 0.1 mM also inhibited the rate of uptake of asialo-fetuin by the cells, and part of the inhibitory effect seen on degradation could thus be secondary to the reduced uptake. However, when chloroquine was added to cells that had taken up asialo-fetuin in advance (and then incubated in asialo-fetuin free medium), degradation of the labelled protein was inhibited almost instantaneously, and this indicated that chloroquine, apart from inhibiting uptake, also retarded intracellular proteolysis of asialo-fetuin.

If the only effect of the weak bases was reduced

intralysosomal proteolysis, accumulation of undegraded labelled protein in the lysosomes would be expected to occur. However, our data do not indicate that the addition of ammonium ions or chloroquine to the cells led to increased amounts of undegraded protein in the lysosome-rich fractions. The fact that both ammonium ions and chloroquine render the lysosomes lighter made it difficult to differentiate between these organelles and endocytic vesicles containing labelled protein when the cells had been incubated with the bases for prolonged time periods (more than 60 min), as the distribution curves for radioactivity and the lysosomal marker enzymes overlapped considerably. Nevertheless, even after 3 hr of incubation with ammonium ions or chloroquine, the major portion of cell-associated radioactivity was clearly not associated with the lysosomes.

Most of the labelled asialo-fetuin in the cells was recovered in structures which, in some types of experiments, were found to behave similarly to the structures that contained the plasma membrane marker enzyme 5′nucleotidase. However, we have several reasons for rejecting the hypothesis that most of the cell-associated asialo-fetuin is bound to the plasma membrane: (1) by determining the amount of asialo-fetuin that is actually bound to the outside surface of the cells when samples of the suspension are removed for homogenization, it may be shown that this amount is an insignificant fraction of the total [2]; (2) very little binding by the receptor occurs in the homogenate, as the concentration of calcium ions is too low (less than 0.05 mM) to allow binding (H. Tolleshaug, unpublished observations); (3) digitonin affects the distribution of 5′nucleotidase and radioactivity differently; and (4) the distributions of radioactivity and 5′nucleotidase were entirely different in fractions obtained by differential centrifuging, provided the cells had been incubated for more than 60 min after the uptake of asialo-fetuin. This was true both for control cells and for cells incubated with chloroquine or ammonium chloride. Accordingly, the coincidence of the distribution curves for radioactivity and 5′nucleotidase is incidental. It is not the consequence of the two activities being contained within the same structure.

The possibility remains that chloroquine and ammonium ions may reduce the rate of entry of endocytosed asialo-fetuin into the lysosomes. This reduction might occur by way of inhibition of transport of endocytic vesicles through the cytoplasm or by inhibition of fusion between endocytic vesicles and lysosomes. As regards inhibition of transport, it has been shown that colchicine reduces the degradation of asialo-fetuin [6], 100 μM colchicine producing a maximal effect. This concentration may safely be assumed to cause complete disruption of the microtubular system. In cells that are exposed to 100 μM colchicine, 50 μM chloroquine or 5 mM NH_4Cl causes proportionally the same reduction in degradation as it does in control cells. Thus we may exclude the possibility that chloroquine and ammonia act on microtubuli.

Chloroquine and ammonium ions may cause a restriction on the movements of the lysosomes and/or the endocytic vesicles. This restriction could reduce the probability of intracellular encounters between these organelles. Furthermore, the probability that fusion will result from such encounters may be reduced if the membranes of one or both kinds or organelles are changed. The uptake of e.g. chloroquine into lysosomes leads to a tremendous increase in the surface area of these organelles. It is not unreasonable to assume that the chemical and physical properties of the additional membrane may be different from that of the normal lysosomal membrane.

If all endocytic vesicles containing asialo-fetuin have the same probability of fusion with lysosomes, then the amount of asialo-fetuin presented to the lysosomes at any given time is a constant fraction of the amount of asialo-fetuin contained in endocytic vesicles. Under this assumption, the amount presented to lysosomes will be at a maximum about 15 min after the start of uptake, when nearly all the asialo-fetuin has been taken up from the medium, but before any significant degradation has taken place.

Another possibility is that the endocytic vesicles go through a 'maturation' process, and that the likelihood of their fusion with the lysosomes increases with time after their formation at the cell membrane. If this is the case, then more and more endocytic vesicles containing asialo-fetuin would fuse with lysosomes—leading possibly to some degree of accumulation of labelled protein in these organelles. Change in the properties of endocytic vesicles is indicated by the observation that their equilibrium density in sucrose gradients increases with time (from 1.12 to 1.16).

However, even if NH_4^+ and chloroquine inhibit, directly or indirectly, the fusion between phagosomes and lysosomes, the degradation of asialo-fetuin was not completely abolished with the concentrations of the bases used. Some asialo-fetuin must therefore still gain access to the lysosomes. That the degradation of asialo-fetuin which still goes on in the presence of chloroquine or NH_4^+ really takes place in the lysosomes is indicated by the finding that leupeptine, an inhibitor of the lysosomal protease cathepsin B [20], inhibits degradation additively (unpublished observations).

Using microscopy to study the rate of fusion between lysosomes and phagosomes containing yeast cells in macrophages, D'Arcy Hart concluded that chloroquine promotes rather than inhibits fusion between phagosomes and lysosomes [21]. There are a number of prominent differences between his experimental system and ours, including time scale, concentration of chloroquine used, type of endocytosis, type of cell studied, and method of measurement. Chloroquine may well have an opposite effect in these two cases.

In conclusion, the effects of ammonia and chloroquine on the degradation of asialo-fetuin in isolated rat hepatocytes are compatible with the idea that the asialo-glycoprotein is taken up into the cells by adsorptive endocytosis, that it accumulates primarily in endocytic vesicles, and that it is digested in the lysosomes of these cells. The lysosomal degradation is, however, so effective that it was not possible to demonstrate labelled protein associated with these

organelles until about 45 min after the uptake of the protein had started. The reason for the 'late' accumulation of labelled protein in the lysosomal fractions is at present somewhat obscure. The bases sharply reduce the amount of accumulated protein in the lysosomes, and this is explained by a reduced rate of fusion between endocytic vesicles and lysosomes.

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