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INHIBITION OF SOME SPONTANEOUS SECRETORY PROCESSES IN MACROPHAGES AND FIBROBLASTS BY AMMONIUM CHLORIDE

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Abstract—Ammonium chloride inhibits the spontaneous secretion of lysozyme by mouse macrophages and the murine macrophage-like cell line P388D_1 and the spontaneous secretion of lysosomal enzymes by P388D_1 cells, normal human fibroblasts and the hypersecretory mucolipidosis II (I-cell) fibroblasts. NH_4Cl can also inhibit protein synthesis, but this is shown to occur mainly at higher concentrations, or after longer periods of exposure, than are needed for the inhibition of the spontaneous secretory processes. It is confirmed that this amine can also interfere with the continuous endocytosis of fluid in the murine cell types. The nature of the inhibitions is discussed.

Amines, and particularly NH_4Cl and chloroquine, have a variety of effects on processes involved in endocytosis and intracellular vesicle fusion. For instance, clustering of ligand–receptor complexes (discussed critically in [1, 2], endocytosis of plasma-membrane bound molecules and of fluid [3, 4], and fusion of vesicles containing particles with secondary lysosomes [5] can all be inhibited by amines. We have critically reviewed these phenomena [6].

The mechanisms underlying these effects are unclear. One possible component is the raised pH in intracellular vesicles which are normally of low pH (such as lysosomes, and also at least some newly formed endocytic vesicles [7, 8]). This rise seems to result from the weak base nature of many amines, which allows their accumulation in vesicles of low pH, with consequent elevation of the pH within the vesicle [9, 10]. Two examples of the likely consequence of this rise in intravesicular pH are the reduction of virus fusion with the vesicular membrane and exit to the cytosol for replication (as in the case of Semliki forest virus [7]), and inhibition of recycling of receptors for ligands such as alpha-2-macroglobulin, which fail to dissociate from their ligands at elevated pH [11].

A similar prevention of dissociation of the receptor for mannose-6-phosphate termini of lysosomal enzymes (which is concerned with correctly directing newly synthesized lysosomal enzymes to lysosomal vesicles [12]) by elevated vesicular pH seems to occur in certain amine-treated cells. Concomitantly, amines induce secretion of lysosomal enzymes by fibroblasts [12] and macrophages [13]. This initiation of lysosomal enzyme secretion does not necessarily require any increase in nett vesicle flux to the cell surface, since it may be merely the content of such vesicles which is changed.

In view of the inhibitory effects of amines on flux of vesicles from the plasma membrane to the interior of the cell [3, 4], one might expect an analogous inhibition of outward flux (vesicles from within the cells fusing with the cell surface). An early observation consistent with this possibility has been presented by Seglen and Reith [14], who found that output of serum proteins by cultured hepatocytes is inhibited by NH_4Cl. However, this work did not completely distinguish effects on secretion *per se*, from those on synthesis of the secretory products, so that it cannot be clearly deduced whether vesicle flux was affected. More recently, we have shown that amines can inhibit the efflux from fibroblasts of endocytosed fluid [15]. This measurement is not complicated by effects on synthesis of the secretory product.

A few other indications of inhibition by amines of flux of vesicles to the exterior exist. For instance, we have shown that spontaneous lysosomal enzyme secretion by the transformed macrophage line P388D_1 is inhibited by amines [16]. This secretion is of normal enzyme (containing the mannose-6-phosphate ligand [17, 18]), and seems to be due to a lack of the relevant receptor. Thus this amine effect seems not to be on the ligand–receptor interaction, but may be on vesicle flux itself.

Therefore, the purpose of the present investigation was to assess whether inhibitory effects of amines on secretion are widespread, by studying some further secretory systems (both lysosomal and non-lysosomal).

MATERIALS AND METHODS

Materials. Plastic multi-well dishes (24 and 35 mm diameter) were from Linbro (Sterilin, Teddington, U.K.) and Costar (Cambridge, MA), respectively. Tissue culture tubes (16 mm diameter) were from Sterilin. Pig serum was from Gibco Europe. All other sera, tissue culture media and antibiotics were from Flow Labs. (Irvine, U.K.). Sera were inactivated by heating at 56° for 30 min.

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Biochemical reagents were obtained as follows: Triton X-100 and NH_4Cl (analar grade) were from BDH (Poole, U.K.); pyruvate (sodium salt) was from Boehringer Mannheim GmbH (F.R.G.); and *p*-nitrophenyl-2-acetamido-2-deoxy-beta-D-glucopyranoside was obtained from Koch-Light Labs. Ltd. (Colnbrook, U.K.). Other reagents were of the highest grade commercially available.

Culture of cells. Macrophages were obtained by peritoneal lavage of normal Swiss mice (T.O. strain) as previously described [19]. The cells were incubated in 35 mm culture wells in medium 199 (M199) containing 10% (v/v) heat-inactivated pig serum, 100 I.U./ml penicillin and 100 mg/ml streptomycin at 37°, gassed with 5% CO_2 in air. After establishment of the cultures overnight, the medium was changed and the experimental treatments started, as described below.

$P388D_1$ *culture.* This murine cell line was maintained by passage twice weekly in Eagle's minimum essential medium (EMEM) containing 10% heat-inactivated foetal calf serum and antibiotics as above. For secretion experiments, the cells were transferred to 35 mm culture dishes (1.5×10^6 cells in 3 ml medium), and allowed to adhere and spread for 1 hr before the medium was changed and the experimental treatment commenced.

Fibroblast culture. Skin fibroblast lines from normal human subjects and from I-cell disease (mucolipidosis II) patients were obtained as before [20]. They were maintained in monolayer culture in the same medium as the $P388D_1$ cells. For secretion experiments they were harvested by trypsinization and seeded in 24 mm dishes, in 1.5 ml medium. Both normal and I-cell fibroblasts were seeded at 1.5×10^5/culture. The normal cells formed a confluent monolayer after incubation overnight, while the I-cells were grown up to confluence over 3–4 days (with a medium change every 2 days) because a substantial proportion of the I-cells failed to attach. For both cell types the resultant confluent monolayers were washed twice with phosphate-buffered saline and re-incubated in fresh EMEM (1.5 ml).

Measurement of enzyme secretion and cell lysis. Cells were incubated for the specific periods and then harvested for enzyme assays. In the case of fibroblasts and $P388D_1$ cells, the medium for the secretion period was EMEM containing 10% (v/v) alkaline-inactivated (pH 9.0, 56°, 30 min) foetal calf serum with antibiotics. The alkaline treatment destroys most endogenous serum lysosomal enzyme activities, facilitating, measurement of the activities secreted by cells into the medium, but does not affect the kinetics of lysosomal enzyme secretion by the fibroblasts or $P388D_1$ cells. At the end of the incubation periods the media were removed, and the cells harvested by the addition of 0.1% Triton X-100 in phosphate-buffered saline, followed by scraping with a rubber policeman. Control cultures without cells were studied in parallel to allow measurement of, and correction for, residual enzyme activities in the media themselves. In experiments with mouse macrophages, the highest concentration (50 mM) of NH_4Cl used caused noticeable detachment of the cells from the culture surface, although it was not lytic: for such experiments only, the medium fraction was centrifuged, and the supernatant transferred to fresh tubes, while the pellet (containing the detached cells) was pooled with the lysed cells scraped off the culture dish. The lysosomal glycosidase, beta-*N*-acetyl-D-glucosaminidase ('hexosaminidase') was used as a marker for lysosomal enzyme release into the medium, and this release was shown to be secretion by routinely comparing it with the release of the cytosolic enzyme lactate dehydrogenase [21], which is retained by the cells unless they undergo lysis. Control experiments showed that NH_4Cl (up to 50 mM) had no effect on the stability at 37° in media of hexosaminidase and LDH released by all four types of cell, or on lysozyme from the two types of mononuclear phagocyte. The assays were conducted shortly after harvesting the cultures because of the instability of LDH which can otherwise lead to considerable underestimation of cell lysis. All cell types remained viable in media containing NH_4Cl concentrations up to 50 mM. Percentage release of LDH in representative 24 hr experiments were: fibroblasts; 50 mM NH_4Cl: $6.26 \pm 1.33\%$ and 100 mM NH_4Cl: 5.48 ± 1.37; macrophages; 50 mM NH_4Cl: $9.63 \pm 6.33\%$. For $P388D_1$ cells the highest concentration of NH_4Cl which was used in the secretion experiments was 30 mM: this gave a representative LDH release of $3.0 \pm 0.6\%$ at 24 hr. In all the experiments in which secretion was investigated (up to 48 hr), the LDH release was always less than 13%. In the experiments in which protein synthesis by these cells was investigated, no indication of any increased lysis of cells in the presence of 50 mM NH_4Cl was obtained (judging by the retention of labelled macromolecules by the cells) but the LDH release in these conditions was not investigated. These data indicating that all the cell types under study remain viable in the presence of high concentrations of NH_4Cl are entirely consistent with previous publications [16, 22, 23].

Measurement of protein synthesis. Cells were established by the appropriate protocol above but in tissue culture tubes: 1 ml of peritoneal exudate for macrophages, and 0.5×10^6 $P388D_1$ or 10^5 fibroblasts in 1 ml of their maintenance medium. They were then incubated for various periods up to 24 hr in the same medium with specific additions of NH_4Cl and with 1 μC/ml[1-^{14}C]leucine. The media all contained substantial quantities of non-radioactive leucine, so it was not considered necessary to add further cold leucine in order to establish a constant specific radioactivity in the system. In some experiments the inhibition of protein synthesis by cycloheximide (0.5–5 μg/ml) was studied: cells were preincubated at 37° for 30 min with cycloheximide before the medium was changed and replaced by fresh medium containing the same concentration of cycloheximide with the addition of radioactive leucine. The incorporation of [1-^{14}C]leucine into macromolecular (precipitable by 5% trichloracetic acid) materials was measured as follows: after the incubation, the medium was removed to separate tubes, and the cells were lysed in 1 ml of 0.1% Triton X-100. Bovine serum albumin (200 μl, 10%) was added to the cell fractions as carrier during the subsequent protein precipitations. Both cell and medium fractions were then treated identically: 1 ml of 10% trichloroacetic

acid (TCA) containing 10 mM leucine (cold) was added, and the tubes were centrifuged (5000 g × 10 min). The supernatants were removed and discarded, and the pellets resuspended in 0.5 ml of 5% (w/v) TCA containing 10 mM leucine. The tubes were recentrifuged, and the procedure repeated a total of four times (which was shown to reduce the radioactivity in the discarded supernatants to background levels). The final pellets were dissolved in 0.5 ml formic acid, and counted in a Triton–toluene scintillant (Packard 299) using an automated quench correction system on the Packard Tricarb instrument.

Pinocytosis. Fluid phase pinocytosis by mouse macrophages was measured as before [24] using [^{3}H]sucrose as tracer in the presence of 0.01% w/v carrier sucrose (non-radioactive). Endocytosis is expressed as Endocytic Indices, corresponding to the volume of culture medium whose contained substrate has been endocytosed by the specified time, per mg cell protein.

Enzyme assays. Beta-*N*-acetylglucosaminidase (EC 3.2.1.30) was assayed at pH 4.5 using the methylumbelliferyl fluorogenic substrate for fibroblasts [25] and the *p*-nitrophenyl substrate for mononuclear phagocytes [19]. The activities are expressed as nmole of substrate hydrolysed per hr per culture. The cytosolic enzyme lactate dehydrogenase was measured to allow an estimate of cell lysis during the experiments [19]. A unit of LDH activity converts 1 μmole of substrate per min at 37°. Lysozyme was assayed turbidimetrically as before [21]. One unit causes a reduction in 600*E* of 0.001 per hr. 5.2 μg of hen egg white lysozyme (Sigma) corresponds to 1000 such units of activity.

Measurement of 'rate' of secretion. A difficulty in all studies on the secretion of synthetic products of cells is that cellular concentrations of secretory material may change during the experiment, so that neither absolute nor proportional (released activity as a % of the total activity in the culture system) output of product is necessarily an apt measure of secretory 'rate' (in the specific sense of volume of intracellular vesicle content released to the exterior of the cell per unit time). In the present work, both modes of comparison of secretory activity were used to ensure that changes in output could reasonably be expected to represent changes in secretory rate. In addition, the effect of the experimental conditions on protein synthesis was studied so that at least some conditions in which insignificant effects on synthesis are nevertheless accompanied by significant effects on secretion could be identified.

Expression of results. All data are given as means ± S.D. for groups in which $N = 3$. On the figures, some S.D.s do not exceed the size of the symbol. Results are from single experiments which are representative of two to six independent experiments.

RESULTS

Protein synthesis in cells exposed to NH_4Cl *and cycloheximide*

It has been noted previously [26] that protein synthesis in hepatocytes can be inhibited by NH_4Cl; this effect can be overcome in some circumstances by the addition of cellular metabolites. In contrast, Amenta and Brocher [27] observed no effect of 10 mM NH_4Cl on fibroblast protein synthesis. We have studied the effect of NH_4Cl (0–50 mM) on protein synthesis in all four cell types used here (mouse macrophages, mouse P388D$_1$ cells, human normal fibroblasts and human mucolipidosis II [I-cell] fibroblasts). Table 1 shows some of the data and indicates that at 20–50 mM NH_4Cl protein synthesis measured over 24 hr was significantly inhibited in all four cell types. Measurements were also made in 4 hr incubations, with similar results. Table 1 represents the effect on total synthesis of protein (the sum of the cellular and secreted fractions), but the effects on the two fractions considered separately were also quite similar (data not shown). At 10 mM, inhibitory effects were negligible or very slight. In the case of mouse macrophages and P388D$_1$ cells, we also observed that the inhibitory effect on protein synthesis of 20 μM chloroquine was comparable to that of 50 mM NH_4Cl, as is commonly observed in other respects [10]. Thus experiments using NH_4Cl concentrations of 10 mM or less can be useful in identifying specific effects on secretion which do not depend on drastic reduction in protein synthesis in cells; though it must be pointed out that there is no information as to whether the synthesis of lysosomal proteins is affected in the same way as total protein synthesis. However, data shown later indicate that high concentrations of NH_4Cl not only suppress protein synthesis, but also cause a reduction in the total

Table 1. Inhibition of protein synthesis by NH_4Cl

	Leucine incorporation as % control				
	Amine concentration (mM)				
Cell type	1 mM	5 mM	10 mM	20 mM	50 mM
Mouse macrophages			110 ± 11.4	89.4 ± 3.9	47.2 ± 0.9
Mouse P388D$_1$ cells	101.1 ± 3.6	96.0 ± 4.8	93.1 ± 6.1	91.7 ± 5.5	74.3 ± 3.6
Human normal fibroblasts			93.5 ± 9.1	75.6 ± 11.7	55.9 ± 14.5
Human I-cell fibroblats			90.0 ± 4.1		69.4 ± 7.1

Total incorporation of radioactive leucine into TCA-precipitable molecules in the medium + cells was measured as described in Materials and Methods, during 24 hr incubations. Incorporation is expressed as a percentage of the appropriate control without NH_4Cl. Experiments were not done for the omitted conditions.

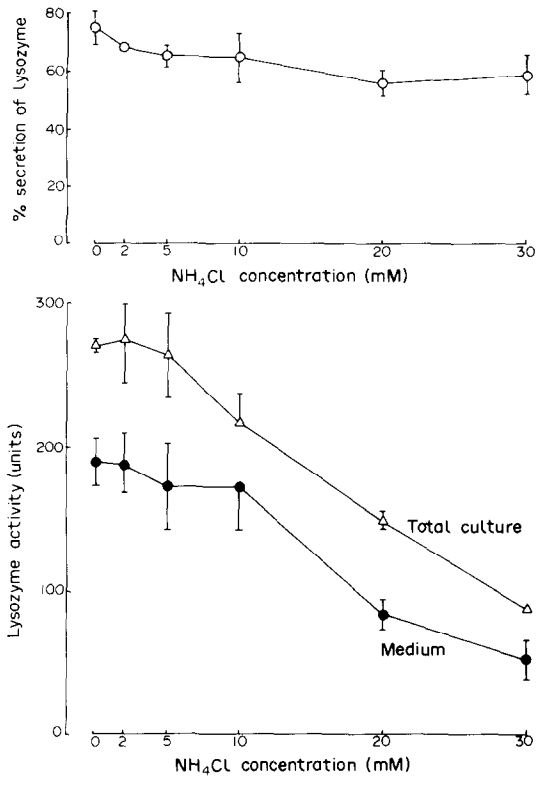

Fig. 1. Lysozyme activities and secretion in mouse peritoneal macrophage cultures exposed to NH_4Cl. % Secretion (○; top section); total culture lysozyme (△) and total medium lysozyme (●). Secretion was measured over 48 hr as described in Materials and Methods.

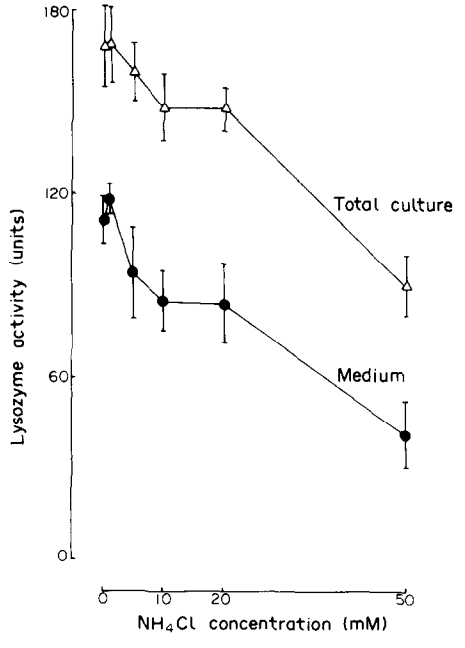

Fig. 2. Lysozyme activities and secretion in mouse $P388D_1$ cells exposed to NH_4Cl for 24 h. Total culture activities (△); total medium activity (●). The experiment was conducted as described in Materials and Methods.

amounts of hexosaminidase in the cultures, as might result from a similar suppression of lysosomal enzyme synthesis. We cannot completely allow for the effects of the amine on protein degradation but in the present cell types the inhibition of degradation is quite small [23], unlike that in hepatocytes [26].

Cycloheximide inhibited protein synthesis powerfully in all cell types: greater than 90% inhibition was always achieved by 2 μg/ml cycloheximide. In the case of mouse macrophages, such a concentration was rather lytic (29.0 ± 6.7% LDH release at 24 hr), so 0.5 μg/ml had to be adopted: it gave >80% inhibition of synthesis at 4 and 24 hr, together with LDH release not different from that of control cultures (9.7 ± 0.9% at 24 hr).

Secretion of lysozyme by mononuclear phagocytes

Lysozyme is secreted spontaneously by mononuclear phagocytes [28], including both mouse macrophages and $P388D_1$ cells [23]. As shown in Fig. 1, NH_4Cl reduced both absolute and proportional output of lysozyme by macrophages, indicative of an effect on secretory rate. Concentrations above 5 mM also depressed total lysozyme activity in the system.

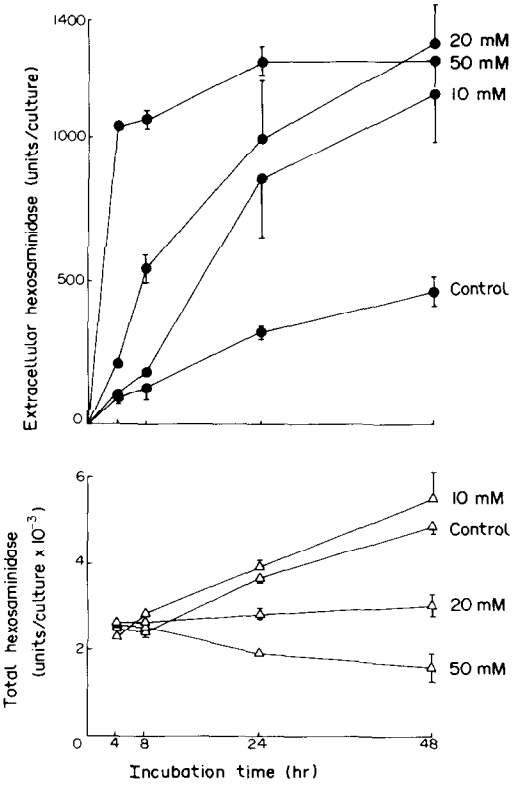

Fig. 3. The induction by NH_4Cl of mouse macrophage hexosaminidase secretion. (a) Secretion; (b) total culture hexosaminidase activity. The experiment was as described in Materials and Methods. Since 50 mM NH_4Cl caused increased detachment of the cells, all media were centrifuged so that these cells could be added back to their respective cell fractions. S.D.s are not shown for the 4 and 8 hr total activities (all these points were indistinguishable statistically) or when they do not exceed the size of the symbol.

Figure 2 shows that a comparable effect was also obtained with $P388D_1$ cells: concentrations of NH_4Cl of 10 mM and higher depressed output, and also total culture activity. Similar observations were made in more extended experiments (48 hr) also.

Lysosomal enzyme secretion by mononuclear phagocytes

We have previously described the induction of hexosaminidase secretion from mouse peritoneal macrophages, and the inhibition of spontaneous secretion by $P388D_1$ cells by a variety of amines [16]. The data on protein synthesis above indicate that effects at concentrations of 10 mM NH_4Cl or less are likely to be effects on secretion *per se*. In addition, Fig. 3 shows that in macrophages, the secretory stimulus provided by 50 mM NH_4Cl was largely confined to the first 8 hr of exposure, after which the release of hexosaminidase proceeded at a similar slow rate in both control and stimulated cultures. The burst was slower and longer-lasting in the presence of 10 and 20 mM NH_4Cl, but after 24 hr, release in these conditions was similar to that in control cultures, and may be due to cell death [21]. Total hexosaminidase activity rose during these experiments, and the rise was unaffected by 10 mM but prevented by 20 mM and reversed by 50 mM NH_4Cl. Figure 4 shows that the burst of release induced over the first 8 hr by 50 mM NH_4Cl was not inhibited at all by 0.5 μg/ml cycloheximide (which inhibits protein synthesis by more than 80%). Figure 4 also gives an example of the comparison of hexosaminidase and LDH release which we made routinely to show that selective secretion, and not lysis, was responsible for hexosaminidase release. Similarly, the longer burst of release induced by lower concentrations of NH_4Cl was not affected by cycloheximide (data not shown). The burst therefore does not require protein synthesis, whereas there is such a protein synthesis-dependent component of fibroblast secretion [29]. An important corollary of the observations of Fig. 3 is that macrophage lysosomal enzyme secretion is not necessarily dependent on protein synthesis, and thus inhibitory effects of low concentrations of the amines on secretion [16] would more likely be direct, rather than indirect, effects operating via protein synthesis.

However, no significant inhibitory effects on macrophage secretion could be detected in conditions in which protein synthesis was relatively unaffected or even at higher amine concentrations, where protein synthesis and total culture hexosaminidase were significantly depressed (data not shown). In the case of $P388D_1$ cells, inhibition of secretion by 10 mM NH_4Cl was detectable even after only 4 hr of exposure, and was prolonged (Fig. 5). It was maximal at 10 mM [10], at which concentration protein synthesis was normal, and culture activity of hexosaminidase was unaffected (data not shown). Thus the inhibitory effects on $P388D_1$ lysosomal enzyme release seem likely to be selective and not secondary to effects on protein synthesis; the inductive effect of amines on peritoneal macrophage secretion precludes a satisfactory determination of

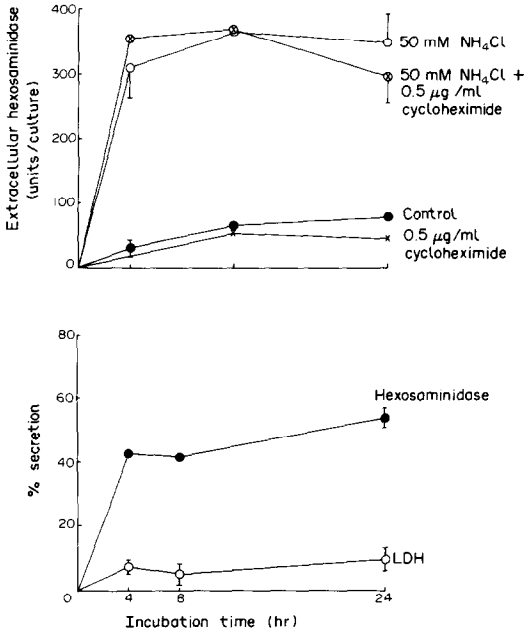

Fig. 4. The effect of cycloheximide on the NH_4Cl secretion by mouse peritoneal macrophages. Cycloheximide was used at 0.5 μg/ml without preincubation; NH_4Cl was at 50 mM. The drop in medium activity in the presence of both agents at 24 hr was noted in several experiments, but is not understood. It may be a technical artefact, but is not simply due to decreased stability of the released enzyme in these conditions, as judged by appropriate control experiments. (a) Hexosaminidase release into the medium. Cells were incubated in control conditions (●); with cycloheximide (×); with NH_4Cl (○); or with both cycloheximide and NH_4Cl (⊗). (b) Release of hexosamindase (●) and lactate dehydrogenase (○) into the medium (as % of the total activity of each enzyme in the whole culture) in the presence of both cycloheximide and NH_4Cl. This was the most lytic of the experimental conditions used in this paper (all other lactate dehydrogenase releases in this experiment were < 10%).

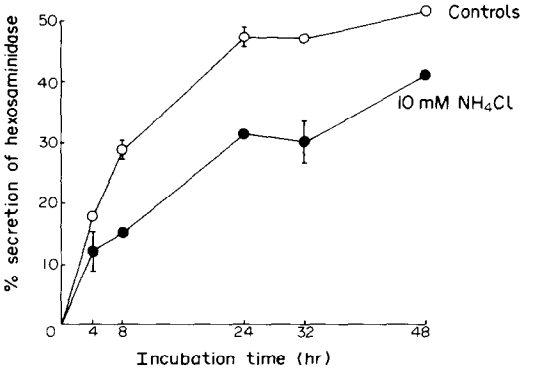

Fig. 5. Inhibition of $P388D_1$ spontaneous lysosomal enzyme secretion by 10 mM NH_4Cl. This was performed as described in Materials and Methods. Hexosaminidase total activities were rising throughout the experiment, and were significantly depressed by the amine only after 8 hr [e.g. at commencement the control (○) and NH_4Cl (●) cultures contained 1533 ± 45 and 1533 ± 110 nmole/hr of hexosaminidase respectively, while the corresponding values at 48 hr were 26011 ± 318 and 20988 ± 314].

whether or not a similar effect occurs in that cell type.

Lysosomal enzyme secretion by human fibroblasts

The secretory response of human fibroblasts to NH_4Cl is maximal at much lower concentrations (10 mM) than in the case of macrophages. Thus at higher concentrations of amines, the onset of an inhibitory effect on secretion (both in absolute and % terms) was detectable (Fig. 6). However, it must be noted that at high NH_4Cl concentration total culture hexosaminidase activity is slightly depressed. Nevertheless, the data are suggestive of an inhibitory component of the action of the amines on secretion, which might well represent an inhibition of vesicle flux.

The data on I-cell secretion implicate further such an inhibitory effect (Fig. 7). These cells are constitutively incapable of synthesis of the mannose-6-phosphate ligand of lysosomal enzymes, and thus their lysosomal enzymes are continuously misdirected, so that the majority of the activity is secreted, and the cells themselves are deficient in intracellular activity of a wide range of lysosomal enzymes [12]. NH_4CL (10 mM) had no effect on the spontaneous secretion in experiments up to 24 hr. But 50 mM NH_4Cl (Fig. 7) inhibited secretion (as assessed in both ways) within 4 hr of exposure. While this concentration of the amine did inhibit protein synthesis (Table 1), it only caused a depression in total culture activity of hexosamindase after exposure of 8 hr or more. Thus the onset of the inhibitory effect on secretion may precede the onset of effects on protein synthesis, and specifically those on the cellular levels of the lysosomal hydrolase. The inhibition of secretion thus should indicate a reduction in vesicle transport to the cell surface.

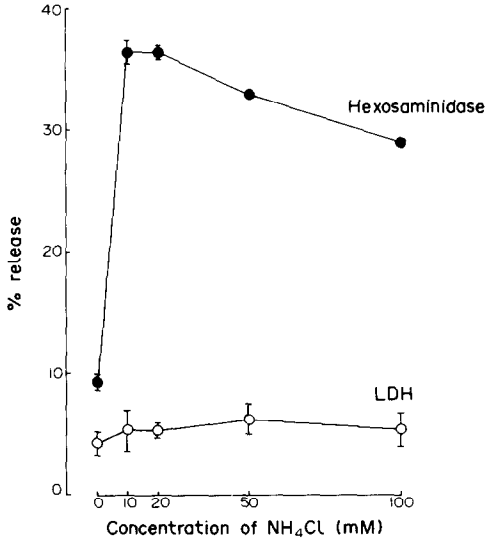

Fig. 6. Inhibition by NH_4Cl of spontaneous lysosomal enzyme secretion by human fibroblasts. Secretion of hexosaminidase (●) was measured at 24 hr with various concentrations of amine, and is compared with release of lactate dehydrogenase (○). Total culture activities of the two enzymes were not perturbed by 10 or 20 mM NH_4Cl, but LDH was depressed at 50 and 100 mM (614 ± 73 and 644 ± 58 units/culture respectively, compared with controls at 750 ± 44); while hexosaminidase was slightly depressed only at 100 mM (198.6 ± 2.1 nmole/hr per culture compared with controls of 229.2 ± 6.1).

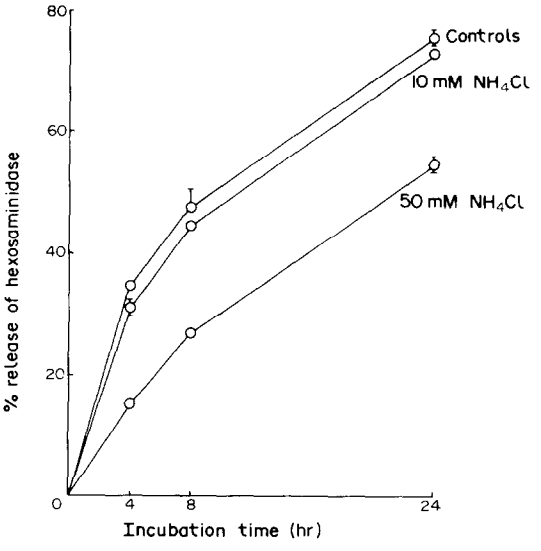

Fig. 7. Inhibition by NH_4Cl of spontaneous lysosomal enzyme secretion by I-cell fibroblasts. Confluent I-cell monolayers in 24 mm Linbro culture wells were exposed to control conditions, or to NH_4Cl at 10 or 50 mM for up to 24 hr, for measurement of secretion as in Materials and Methods. Total hexosaminidase in the cultures was not depressed by 10 mM NH_4Cl, and only by 50 mM after 24 hr (controls: 424 ± 16 nmole/hr per culture; 50 mM: 378 ± 17).

Pinocytosis in macrophages

Since the previous evidence [3, 4] of inhibition of fluid phase pinocytosis by NH_4Cl has not been obtained with the cells we have used here, we have sought to demonstrate such inhibition in mouse peritoneal macrophages and $P388D_1$ cells to complement the rest of our data. In macrophages, uptake of fluid was not linear for more than 6 hr, as noted previously [24]. In the case of $P388D_1$ cells, pinocytosis was not linear (Table 2). Table 2 shows that the uptake of fluid by both cell types was significantly inhibited by NH_4Cl even at 1–5 mM, and in a dose-dependent fashion. It is difficult to estimate the degree of inhibition in view of non-linear kinetics, but if the zero-time fluid uptake (which represents entrapment in the case of a non-adsorptive tracer such as sucrose) is subtracted from all the values, then maximal inhibition is apparent at the earliest times (*ca* 45% for macrophages in 10 mM amine, and for $P388D_1$ in 20 mM, at 2 hr). Using the same computation, the degree of inhibition at 24 hr (macrophages) and 48 hr ($P388D_1$ cells) has declined to *ca*15 and 20%, respectively. This is consistent with the previous work in different cell and tissue systems [3, 4], in that rather high degrees of inhibition were reported (up to 80% in [4]) in short-term experiments (up to 3 hr). The inhibition in the present studies was established within 2 hr, and so its reversibility

Table 2. Inhibition of pinocytosis in mononuclear phagocytes by NH_4Cl

Cell type	Condition fluid uptake (μl/mg cell protein)					
		Duration of pinocytosis				
		0 hr	2 hr	6 hr	24 hr	48 hr
Mouse Macrophage	Control	0.2 ± 0.02	0.42 ± 0.06	1.25 ± 0.09	4.3 ± 0.1	
	5 mM NH_4Cl		0.36 ± 0.01	1.10 ± 0.03	3.8 ± 0.1	
	10 mM NH_4Cl	0.21 ± 0.1	0.32 ± 0.1	0.97 ± 0.01	3.65 ± 0.2	
$P388D_1$	Control	0.29 ± 0.3	0.80 ± 0.05	1.98 ± 0.1		16.8 ± 0.3
	1 mM NH_4Cl		0.71 ± 0.03	1.8 ± 0.2		16.5 ± 0.1
	5 mM NH_4Cl		0.63 ± 0.1	1.83 ± 0.1		13.4 ± 0.4
	10 mM NH_4Cl		0.60 ± 0.1	1.7 ± 0.1		13.7 ± 0.7
	20 mM NH_4Cl	0.23 ± 0.1	0.56 ± 0.03	1.61 ± 0.18		13.4 ± 0.7

Pinocytosis was measured as described in Materials and Methods. Zero time uptake was determined by adding the labelled sucrose for 5 min before washing the cells and processing as normally. Experiments were not done for the omitted conditions.

was tested with macrophages exposed for 2 hr to 0–20 mM NH_4Cl. When such cells were washed four times with PBS they re-established control rates of pinocytosis: uptake in the subsequent 8 hr was statistically indistinguishable in all groups (the control group had endocytosed sucrose corresponding to 1.2 ± 0.1 μl/mg protein). The inhibition of $P338D_1$ pinocytosis was also completely reversible (data not shown). The reversibility of the inhibition of pinocytosis in both cell types and also gives further indication that the inhibitory effects of NH_4Cl on secretion by these cells is unlikely to result from irreversible metabolic damage to the cells.

DISCUSSION

The data above indicate clearly that secretion of lysozyme by two types of mononuclear phagocyte can be reduced by NH_4Cl; that spontaneous lysosomal enzyme secretion by $P388D_1$ cells can be similarly affected; and imply that the same may be true of the low rate spontaneous secretion of lysosomal hydrolases by normal fibroblasts, and the high rate secretion of these molecules by I-cells. Thus a range of spontaneous secretory processes can be retarded by NH_4Cl (cf. also [14]). At least in the case of lysosomal enzyme secretion by $P388D_1$ cells, a variety of other amines are comparably effective [16]. It seems very likely that these inhibitions all involved a reduction of intracellular vesicle transport to, and fusion with, the plasma membrane.

Similarly, our evidence confirming the inhibition of fluid phase pinocytosis by NH_4Cl also implies a reduction of membrane fusion at the plasma membrane, unless one can envisage the formation of vesicles which largely exclude fluid. The large degree of inhibition observed at the earliest times may be partly a consequence of modification of fluid efflux, since this is proportionally very rapid immediately after supply of a radioactive fluid tracer [15, 30]. But the later inhibition is unlikely to depend on changed vesicle efflux, since at the concentrations in question NH_4Cl inhibits output [15], which would lead to an underestimation of inhibition of uptake. While the mechanisms by which the amines affect vesicle flow remain unclear, it is possible that the central feature they share, membrane fusion, is the site of action.

It is notable that amines affect several other systems in which synthesis of a macromolecular agent is closely coupled to its transport either to the cell surface or to the exterior. An example of the former, transport to the cell surface, is the production of the procoagulant thromboplastin by human monocytes in culture: this process is enhanced by many amines and the enhancement requires protein synthesis [31]. But whereas monocytes normally carry nearly all their thromboplastin apoprotein to the plasma membrane, where it is available on the external surface, amine-treated cells seems to retain a larger amount within cells [31]. Since large nett increases in the amount of the procoagulant present in the cells occur during these experiments, and depend on protein synthesis, it seems unlikely that effects of the amines on bulk protein synthesis could be responsible for the reduction in thromboplastin externalization, which may again involve a reduced vesicular transport to the cell surface. The mechanism of induction of enhanced culture levels of thromboplastin is not yet known.

An example of the latter systems, in which synthesis is coupled to secretion, is plasminogen activator, the non-lysosomal neutral proteinase. At least in mouse macrophages, this enzyme is hardly present within cells, and when its production is stimulated by a variety of agents, the majority is rapidly transported out of the cell [28]. We have recently found [22] that NH_4Cl initiates such output of plasminogen activator by mouse peritoneal macrophages, and enhances the spontaneous output by $P388D_1$ cells. Again the mechanism of the enhancement is unclear. But while the intracellular transport of the molecule has yet to be studied, we have noted that at low concentrations of the amine (*ca* 1 mM) there is an inhibitory action on $P388D_1$ plasminogen activator production (Roberts, Jessup and Dean, unpublished data). This may again be an indication of an inhibition of the vesicular transport component of plasminogen activator output. Thus in the cases of thromboplastin and plasminogen activator there may be at least two coexisting components of the action

of the amine which predominate at different concentrations: both a stimulation of production and an inhibition of transport.

The independence of the first phase (burst) of lysosomal enzyme secretion by macrophages from protein synthesis (Figs. 3 and 4) indicates an important distinction between this mechanism and those of thromboplastin, plasminogen activator and lysozyme [28] production and release, since the latter do require protein synthesis. Consistent with this view, Gee *et al.* [32] have found that rifampin, an inhibitor of protein synthesis, suppresses the output of lysozyme and elastase (a non-lysosomal neutral proteinase) but does not affect lysosomal beta-glucuronidase release in thioglycollate-induced mouse peritoneal macrophages. Thus the inhibition of lysosomal enzyme secretion from the mouse macrophage-like cell line reported here is likely to be an effect on vesicular flow.

The dependence of several other transport and secretory processes on concurrent protein synthesis tends to obscure any direct effect of amines (which may inhibit protein synthesis) on the transport itself. Nevertheless, there is increasing evidence of the inhibition of at least *spontaneous* secretory events by amines. It is experimentally unfortunate that the triggered secretions and transport processes (lysosomal enzyme and plasminogen activator release, and thromboplastin production, by mononuclear phagocytes) with which we have previously been concerned are themselves also triggered by amines. For this precludes the clear-cut demonstration as to whether such triggered secretions can also be inhibited by amines. This question requires attention in other suitable systems which are not also initiated by amines. In preliminary work (Dean and Gomperts, unpublished data) we have found that mast cell histamine degranulation is not initiated by NH_4Cl and certain other amines; thus in this system it will be possible to answer this question.

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