Effects of Ammonia on Processing and Secretion of Precursor and Mature Lysosomal Enzyme from Macrophages of Normal and Pale Ear Mice: Evidence for Two Distinct Pathways

JOHN A. BROWN, EDWARD K. NOVAK, and RICHARD T. SWANK
Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, New York 14263. Dr. Brown's present address is Department of Obstetrics and Gynecology, University of Washington, Seattle, Washington 98195.

ABSTRACT   Lysosomal enzymes have been shown to be synthesized as microsomal precursors, which are processed to mature enzymes located in lysosomes. We examined the effect of ammonium chloride on the intracellular processing and secretion of two lysosomal enzymes, β-glucuronidase and β-galactosidase, in mouse macrophages. This lysosomotropic drug caused extensive secretion of both precursor and mature enzyme forms within a few hours, as documented by pulse radiolabeling and molecular weight analysis.

The normal intracellular route for processing and secretion of precursor enzyme was altered in treated cells. A small percentage of each precursor was delivered to the lysosomal organelle slowly. Most precursor forms traversed the Golgi apparatus, underwent further processing of carbohydrate moieties, and were then secreted in a manner similar to secretory proteins. The lag time for secretion of newly synthesized β-galactosidase precursor was notably longer than that for the β-glucuronidase precursor. The source of the secreted mature enzyme was the lysosomal organelle.

Macrophages from the pale ear mutant were markedly deficient in secretion of mature lysosomal enzyme but secreted precursor forms normally.

These results suggest that ammonia-treated macrophages contain two distinct intracellular pathways for secretion of lysosomal enzymes and that a specific block in the release of lysosomal contents occurs in the pale ear mutant.

Ammonium chloride prevents the intracellular processing of lysosomal enzyme precursors to mature forms and increases the rate of secretion of precursor forms in fibroblasts (1). Some of these effects of ammonia may be related to the known accumulation of ammonia and other primary amines in lysosomes (2, 3) and in other acidic organelles (4), with an accompanying increase in organellar pH (reviewed in reference 5). At neutral pH the dissociation of cell surface complexes of lysosomal enzymes and their receptors is slowed (6, 7). Similarly, the amine-mediated increase in intracellular pH is postulated to reduce the level of unoccupied receptors involved in delivery of newly synthesized lysosomal enzymes to lysosomes. Therefore, ammonia may be an important tool

in dissecting the intracellular pathways and routing mechanisms for newly synthesized and receptor-associated lysosomal enzymes.

Recent experiments with resident mouse peritoneal macrophages (8–10) demonstrated a striking effect of ammonium chloride and other primary amines; namely, a much greater and more rapid secretion (>50% in <3 h) of lysosomal enzyme activity than reported for other cell types. We have examined in more detail the effect of ammonia on the intracellular processing and secretion of precursor and mature forms of lysosomal enzymes in thioglycollate-inducted macrophages, which have considerably greater rates of synthesis and intracellular levels of lysosomal enzymes than resident

macrophages. Our results show that stimulated macrophages rapidly secrete not only precursor but also mature forms after treatment with ammonia, in contrast to other cell types in which only precursor is secreted, and that macrophages from the pigment mutant, pale ear, are selectively deficient in secretion of mature form. Also, several novel and in some cases differential effects of the ammonia treatment on intracellular processing, transport, and secretion of β-glucuronidase and β-galactosidase were noted. These studies complement similar ones we have recently completed on untreated macrophages (11–13).

MATERIALS AND METHODS

Cell Culture: Thioglycollate-stimulated macrophages were isolated, cultured, and labeled with [^{35}S]methionine as previously described (12). The source of macrophages were inbred C57BL/6J female mice, unless indicated otherwise. When necessary, ammonium chloride was added to the medium from a 10X stock dissolved in the appropriate medium, titrated to pH 7.4, then resterilized by passage through a 0.2-μm filter. In treated cells, ammonium chloride was present in the methionine-deficient medium in which the cells were incubated for 0.5 h before they were labeled; in the labeling medium; and in any subsequent chase media.

We collected cells by removing the medium, washing the attached cells quickly in fresh medium, then scraping them free in a small volume of appropriate buffer. The medium and wash were centrifuged at low speed and any pellet formed from unattached cells was combined with the rest of the cells.

Purification of Enzymes and Subcellular Fractionation: For isolation of enzyme the cell suspension was first sonicated, Triton X-100 was added to a final concentration of 1%, and a high speed supernatant was prepared (12). β-Glucuronidase and β-galactosidase were purified from the supernatant by the addition of carrier enzyme and monospecific antibody, as previously described (12). Purification of medium enzyme was performed similarly except for omission of the sonication step.

Macrophage homogenization, subcellular fractionation on a gradient formed from 25% Percoll, and enzyme isolation from the resulting fractions using antibody bound to cyanogen bromide–activated Sepharose were done as previously described [13]. For preparation of a total particulate fraction, homogenates were centrifuged successively for 10 min at 120 g; 10 min at 2,400 g; 20 min at 9,800 g; and 30 min at 100,000 g. Each pellet was resuspended in 0.25 ml of 0.25 M sucrose and the combined 1 ml was applied to the Percoll gradient. This procedure was found to reduce considerably the lysis of labile lysosomal organelles that ensued if particulate material was simply isolated by centrifugation for 30 min at 100,000 g. The percentage of lysosomal enzymes that was solubilized and thus presumed to arise from lysed lysosomes varied from 29 to 32% for both normal and ammonia-treated cells after the modified procedure above.

Electrophoresis: The immune complex was solubilized by being boiled in 1% SDS in the presence of β-mercaptoethanol. Samples were electrophoresed on 7% acrylamide gels as described by Laemmli (14). Gels were fixed and impregnated with 2,5-diphenyl oxazole as described by Bonner and Laskey (15), dried, and exposed to Kodak X-omat AR x-ray film. When necessary, the exposed plate was used as a template to isolate the radioactive areas on the dried gels, which were then cut out, and the radioactivity was determined by counting in scintillation fluid as described (12). Radioactivity in total protein was determined after precipitation of 0.01-ml aliquots of cell homogenate with 5% trichloroacetic acid as previously described (16).

Endoglycosidase H Digestions: Radiolabeled immunoprecipitates were dissolved by being boiled for 2 min in 10 μl of 1% SDS in water. To this was added 40 μl of 30 mM sodium citrate buffer, pH 5.5, containing 1.5 mU of endoglycosidase H (endo H).[1] The total mixture was incubated at 37°C for 24 h, dissolved in urea–SDS, boiled, and electrophoresed in one dimensional gels containing urea–SDS (14).

Enzyme Assays: β-Glucuronidase (EC 3.2.1.31) and β-galactosidase (EC 3.2.1.23) enzymic activities were measured spectrofluorometrically (17). Lactic dehydrogenase activity was measured by the method of Reeves and Fimognari (18).

Materials: Female C57BL/6J (+/+) normal inbred mice, C57BL/6J beige (*bg^J/bg^J*), and C57BL/6J pale ear (*ep/ep*) pigment mutants congenic to the C57BL/6J inbred background were raised in our own facilities from stocks

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[1] *Abbreviation used in this paper*: endo H, endoglycosidase H.

originally obtained from the Jackson Laboratories (Bar Harbor, ME). Culture media and sera were obtained from Gibco Laboratories, Inc. (Grand Island, NY). Thioglycollate was obtained from Difco Laboratories Inc. (Detroit, MI). [^{35}S]Methionine and NCS tissue solubilizer were from Amersham Corp. (Arlington Heights, IL). Percoll was supplied by Pharmacia Fine Chemicals (Piscataway, NJ). Reagents for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). X-Omat AR photographic film was from Eastman Kodak Co. (Rochester, NY). Tissue culture flasks were obtained from Corning Glass Works, Corning Science Products (Corning, NY). Endo H was obtained from Health Research, Inc. (Albany, NY). All other chemicals were from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA) and of the highest purity available.

RESULTS

Ammonia Induces Rapid and Extensive Secretion of Lysosomal Enzymes

Ammonium chloride causes massive secretion of both β-glucuronidase and β-galactosidase enzyme activities (Fig. 1). Relatively low concentrations (>3 mM) of ammonium chloride induced significant release of enzymes. Near-maximal release of β-glucuronidase occurred at 10 mM NH₄Cl. β-Galactosidase activity was released approximately in parallel.

It should be noted that, although these are relatively high nominal levels of NH₄Cl, at pH 7.4 and 50 mM NH₄Cl the concentration of the free base, NH₃, which is most probably the active agent in stimulation of lysosomal enzymes release (3, 19), is only 0.7 mM. The release of enzymic activity was not significantly influenced by cell lysis, as only ≤7% of cytoplasmic lactic dehydrogenase was released at 50 mM NH₄Cl. An additional indication that ammonia was not causing general cell toxicity was that the drug caused selective secretion of a form of β-galactosidase that was relatively
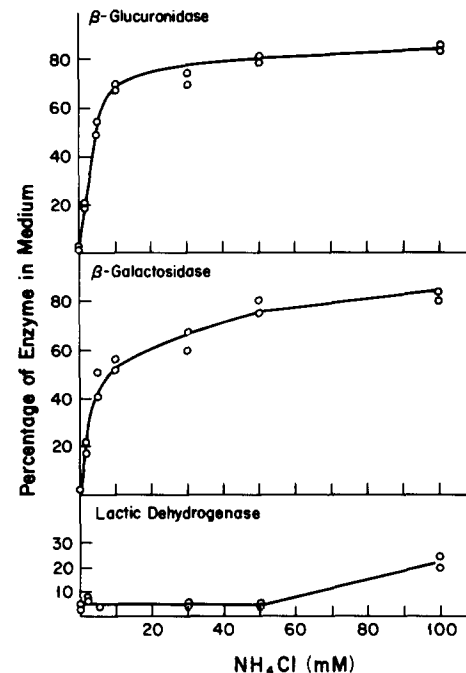

FIGURE 1 Secretion of β-glucuronidase and β-galactosidase activities with increasing concentrations of ammonium chloride. Cultured macrophages were incubated with fresh medium containing varying concentrations of ammonium chloride. After 3 h the medium was removed and the cells were harvested. The medium was centrifuged and any pellet that resulted from detached cells was combined with the harvested cells. Percentage of enzyme in medium is the percentage of total (cellular + medium) released.

resistant to endo H (see Fig. 7). At concentrations of NH₄Cl >10 mM, we observed a change in cell morphology. Cells rounded up and were anchored less tightly to the culture flask. However, cells remained >93% viable after up to 8 h in 50 mM ammonia as measured by cytoplasmic lactic dehydrogenase release. In other experiments it was determined that treatment with 50 mM ammonium chloride caused a 30–50% decrease in the rate of incorporation of [^{35}S]methionine into total macrophage proteins in a 30-min pulse labeling. However, the rate of synthesis of β-glucuronidase and β-galactosidase relative to total macrophage protein synthesis was not affected. In addition, the effects of treatment for 6 h with 50 mM ammonia were reversible in that treated cells immersed in normal media regained the capacity to process precursor forms of lysosomal enzymes to mature forms at <1 h and regained their normal concentrations of intracellular lysosomal enzymes after several additional days in culture. We used 50 mM ammonia in many of the experiments reported here since we found that 10 mM ammonia gave incomplete inhibition of processing of β-galactosidase, and at concentrations <50 mM there was incomplete inhibition of processing of β-glucuronidase.

The release of enzymic activity was rapid, with significant release after 30 min and near-maximal levels (50–60%) of both enzymes being released within the first 2 h after ammonia treatment. No change of total lysosomal enzymic activity (cells + medium) was observed during the relatively short time periods of the experiments in this report.

Most of the Secretable Intracellular Pool Derives from Lysosomes

The internal pool from which these enzymes were released was examined (Fig. 2) by fractionation of extracts by density equilibrium sedimentation on Percoll gradients. Most β-glucuronidase activity is found in a peak at the high density region of the gradient, with a lower density shoulder reaching throughout the gradient. The high density peak is thought to contain the lysosomal organelle since it not only contains the bulk of lysosomal enzyme activity but is also the nearly exclusive site of mature β-glucuronidase and β-galactosidase (13). When cells were treated with ammonium chloride, the bulk loss of enzymic activity was from the lysosomal peak of activity (fractions 11–15). Of the total secretions of β-glucuronidase and β-galactosidase upon treatment with 50 mM NH₄Cl, 66 and 62% respectively were derived from fractions 11–15.

Comparative Secretion of Mature and Precursor Forms

The release of [^{35}S]methionine radiolabeled enzymes was followed by immunoprecipitation with monospecific antibodies from the medium and washed cells separately. Qualitatively, ammonium chloride caused secretion of mature enzyme as evidenced by measured molecular weights of 73,000 and 63,000 for radiolabeled β-glucuronidase and β-galactosidase subunits (12), respectively (Fig. 3). In quantitative terms, radiolabeled mature enzyme was rapidly released (significant release at 0.5 h) into the medium in large quantities (~50% of total by 2 h), which paralleled the release of enzymic activity (see Fig. 1). Similar percentages of total enzyme, assayed as either enzyme activity or radiolabeled mature enzyme, were
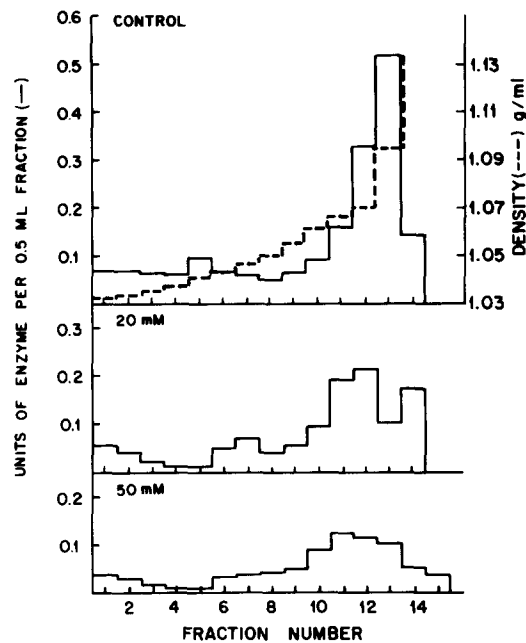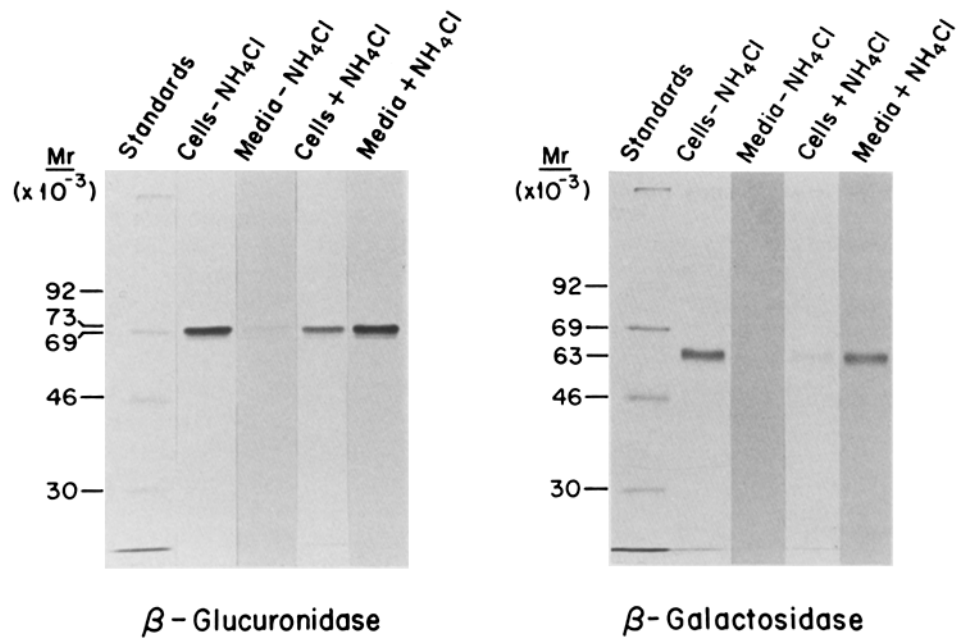
FIGURE 2 Subcellular density distribution of β-glucuronidase in homogenates of normal and ammonium chloride-treated macrophages. Cells were incubated for 3 h in medium containing 0, 20, and 50 mM ammonium chloride. They were then harvested and homogenized in 0.25 M sucrose, and a total particulate fraction was prepared as described in experimental procedures. The particulate fraction was sedimented through a Percoll gradient and 0.5-ml fractions were collected.
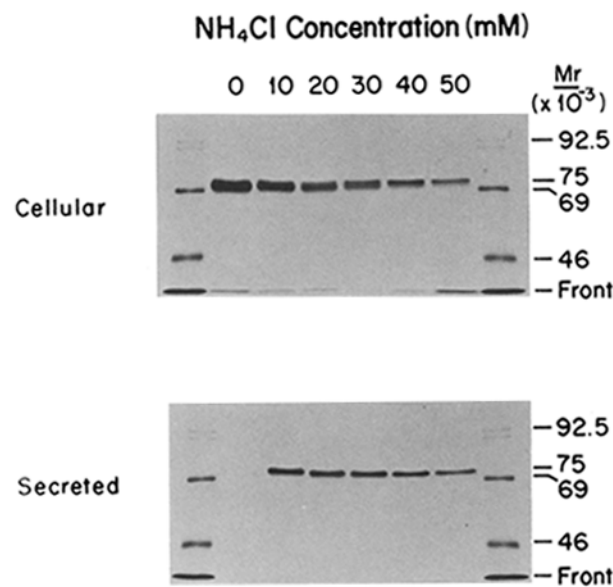
found in the medium at all time points up to 6 h of treatment. The two mature lysosomal enzymes were released at similar rates.

The effect of ammonium chloride treatment on processing and secretion of precursors of lysosomal enzymes in macrophages is in certain respects similar to that observed in other cell types; namely, there is inhibition of processing of precursor forms to mature forms and an elevated rate of secretion of precursor forms (Fig. 4). In untreated cells no detectable secretion of either lysosomal enzyme occurred during the 3 h of this experiment. Also, as expected (12), significant processing of the M_r 82,000 β-galactosidase precursor to the M_r 63,000 mature form had occurred. The small amount of β-glucuronidase precursor present in untreated cells is only 2,000 larger in molecular weight than mature enzyme and is obscured in this figure by the predominant mature enzyme. At 10 mM ammonium chloride processing of β-galactosidase to mature form was reduced and no detectable processing occurred at 20–50 mM. At all concentrations of ammonium chloride, measurable secretion of precursor forms occurred. There is a small decrease in molecular weight (2,000–4,000) for both lysosomal enzyme precursors at ammonium chloride concentrations >10 mM. This is especially evident in the secreted forms. An unusual finding is that β-glucuronidase and β-galactosidase maturation appear differentially sensitive to inhibition in macrophages. There are two forms of cellular β-glucuronidase of only slightly different molecular weight as would be expected for precursor and product forms (12) at 20 and 30 mM ammonia. That processing of β-glucuronidase is relatively resistant to ammonia is supported by other experiments (Fig. 6), which show that the two β-glucuronidase forms have dissimilar subcellular distributions. Imort et al. (20)

FIGURE 3 Evidence that ammonium chloride induces release of mature forms of lysosomal enzymes of macrophages. Macrophages were pulsed with 125 μCi [35S]methionine/25-cm² flask for 1 h, then chased for 24 h with unlabeled medium, so that only mature forms of lysosomal enzymes were radiolabeled. The chase medium was then replaced with fresh medium containing 50 mM ammonium chloride. β-Glucuronidase and β-galactosidase were isolated after 6 h from cell homogenates and medium by sequential immunoprecipitation, electrophoresed on SDS gels and fluorographed. Exposure time was 2 d. Cell homogenates before treatment with ammonia contained 4,300 cpm in purified β-glucuronidase and 1,700 cpm in purified β-galactosidase.

β – Glucuronidase

β – Galactosidase

A β-Glucuronidase

NH₄Cl Concentration (mM)

Cellular

B β-Galactosidase
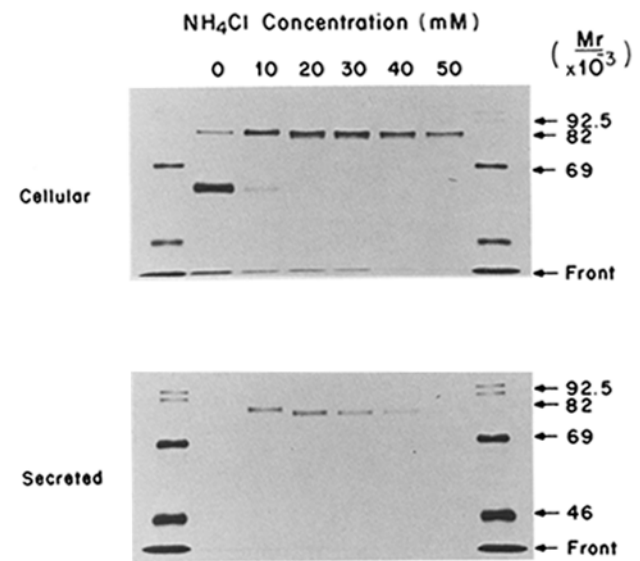
NH₄Cl Concentration (mM)

Cellular

Secreted

Secreted

FIGURE 4 Effect of ammonium chloride on intracellular synthesis, processing, and secretion of precursors of β-glucuronidase and β-galactosidase. Macrophages were pulsed for 15 min with 125 μCi [35S]methionine/25-cm² flask in the presence of various concentrations of ammonium chloride and chased in unlabeled medium of the same composition. After 3 h, the media and cells were harvested separately. β-Glucuronidase (A) and β-galactosidase (B) were sequentially isolated by immunoprecipitation as described, then electrophoresed on SDS gels and visualized by exposure to x-ray plates. The exposure time was 4 d.

likewise observed that 10 mM ammonia causes incomplete inhibition of processing of several lysosomal enzymes in human macrophages.

When we quantified the time course of release of pulse-labeled precursor forms (Fig. 5), we found the two enzymes were released with strikingly different kinetics. For example, at 2 h 25% of β-glucuronidase precursor was secreted as opposed to insignificant secretion of the β-galactosidase precursor. By 6 h the secretion of the two precursor forms was nearly equal at ~40%. This was in sharp difference to the parallel secretion of the mature forms of these enzymes.

Insignificant release (<4%) of either precursor was detected in untreated cells at 3 h chase, in agreement with previous experiments (12).

Subcellular Distribution of Precursors in Ammonium Chloride–treated Cells

When we studied the intracellular distribution of labeled enzymes in ammonium chloride–treated cells, we obtained a result quite different from that for untreated cells reference 13; Fig. 6). At 2 h in the absence of ammonium chloride
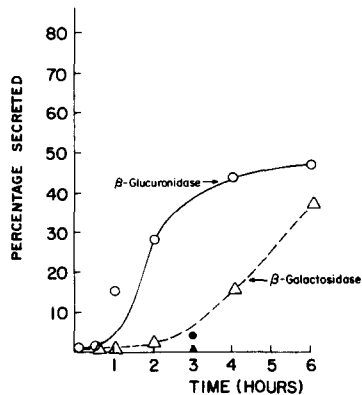
FIGURE 5 Time course of secretion of precursor forms of lysosomal enzymes from macrophages treated with ammonium chloride. Macrophages were labeled with 125 μCi [^{35}S]methionine/25-cm^2 flask (time 0) for 15 min in the presence of 50 mM ammonium chloride, then chased with fresh medium containing the same concentration of ammonium chloride. At the time points indicated the medium was withdrawn from a flask of cells and the cells were harvested. β-glucuronidase and β-galactosidase were purified from each fraction by sequential immunoprecipitation with monospecific antibodies and radioactivity determined as described in experimental procedures. O, β-glucuronidase; △, β-galactosidase; ●, control β-glucuronidase minus ammonia; ▲, control β-galactosidase minus ammonia. There were ~930 cpm in total β-galactosidase and 2,900 cpm in total β-glucuronidase after the 15-min pulse.

~50% of lysosomal enzyme precursors were transferred to the more dense fraction and converted to mature form (13). With ammonium chloride treatment, however, β-galactosidase remaining within the cell stayed exclusively in the precursor form at 2 h and was concentrated in the less dense fractions separated from the bulk of catalytic activity located in lysosomes (Fig. 6 a). With 6 h of ammonium chloride treatment, the radiolabeled enzyme was still entirely in the high molecular weight precursor form. However, the subcellular distribution was now markedly shifted, with significant levels not only in light fractions but also in the more dense lysosomal region of the gradient. No sign of maturation of β-galactosidase was evident in any fraction, even those from the more dense lysosomal part of the gradient.

In contrast, close inspection of Fig. 6 b reveals that there are two forms of β-glucuronidase visible in the ammonium chloride–treated cells at 2 h, consistent with the previously observed (Fig. 4 a) resistance of β-glucuronidase maturation to the inhibitory effects of ammonia. It is interesting that the low molecular weight form is concentrated in the dense lysosomal region of the gradient whereas the high molecular weight form predominates in lighter gradient fractions. Only the low molecular weight form is present at 6 h of treatment and it is concentrated in the more dense fractions. This suggests that the two components of β-glucuronidase are precursor and mature forms and that maturation is ammonia resistant as compared with β-galactosidase maturation.

As an indirect measure of whether secreted forms traverse the Golgi apparatus, we assayed the susceptibility of intracellular and secreted forms of one lysosomal enzyme precursor, β-galactosidase, to endo H (reference 21; Fig. 7). Endo H cleaves oligosaccharides of the high mannose or hybrid type, including those of lysosomal enzymes (22–24), but has no effect on complex oligosaccharides. As expected, intracellular enzyme from untreated cells pulse-labeled for a short time (1

h) with [^{35}S]methionine was highly sensitive to endo H, decreasing in molecular weight from 82,000 to 70,000. When cells were pulsed for 1 h and then chased for 6 h in the presence of ammonia, the maximally deglycosylated 70,000 form was still apparent, but most labeled enzyme was present as a relatively endo H–resistant 70,000-mol-wt form. Most interesting was the fact that enzyme secreted into the media of ammonium chloride–treated cells was entirely the relatively endo H–resistant 77,000-mol-wt form with no 70,000-mol-wt sensitive form visible. Thus the secreted form had some complex oligosaccharides presumably acquired by passage through the Golgi apparatus. We obtained independent evidence that the 70,000-mol-wt form represented maximally deglycosylated β-galactosidase precursor by dual-labeling cells with [^{3}H]mannose and [^{35}S]methionine as described in Fig. 7 and then determining the ratio of [^{3}H]/[^{35}S] in individual components. We found that the [^{3}H]/[^{35}S] ratios were 1.55, 0.61, and 0.024 for the 82,000, 77,000-, and 70,000-mol-wt forms, respectively.

Independent Genetic Control of Secretion of Mature and Precursor Lysosomal Enzymes

One means to test whether secretion of particular lysosomal enzymes occurs via the lysosomal organelle is to examine whether their secretion is altered in mutants abnormal in processing or secretion of the lysosomal organelle. A series of congenic pigment mutants in the mouse fits this description in that they appear to be organellar mutants with defects not only in the cellular apparatus necessary for melanosome formation but also in secretion of kidney (25–28) and platelet (29) lysosomal enzymes and have decreased numbers of platelet dense granules (29, 30).

Macrophages from several of these mutants were screened to determine if secretion of lysosomal enzymes in the presence of ammonium chloride was altered. Beige macrophages secreted β-galactosidase similarly to macrophages of C57BL/6J mice, but macrophages from the mutant pale ear showed a several-fold–decreased secretion of β-galactosidase and β-glucuronidase in the presence of several concentrations of ammonium chloride (Table I). At concentrations of ammonium chloride that caused normal cells to secrete ~50–60% of their lysosomal enzymes, macrophages from pale ear mice secreted only 10–20% of their lysosomal enzyme content. Lactate dehydrogenase secretion was <5% in both normal and mutant mice in the course of this experiment.

We examined the release of labeled precursor and mature enzyme from macrophages of pale ear mice to determine the specificity of the mutant alteration. We found that if the cells were labeled and chased in the presence of ammonium chloride, they secreted precursor enzymes similarly to the C57BL/6J-derived normal cells (Fig. 8 and Table II). However, if the cells were treated so that all radiolabel was in mature lysosomal enzyme forms before treatment with ammonium chloride, then little labeled enzyme (8–9%) was secreted from the pale ear macrophages, whereas 30–40% of the label was secreted in C57BL/6J macrophages. Also, reduced secretion from pale ear macrophages was accompanied by normal or increased levels of intracellular enzymes, indicating that aberrant intracellular turnover cannot account for decreased secretion. The secretion from pale ear macrophages was actually <8–9% since these figures represent maximal secretion, uncorrected for the small amount of secretion (≤5%), from untreated macrophages.
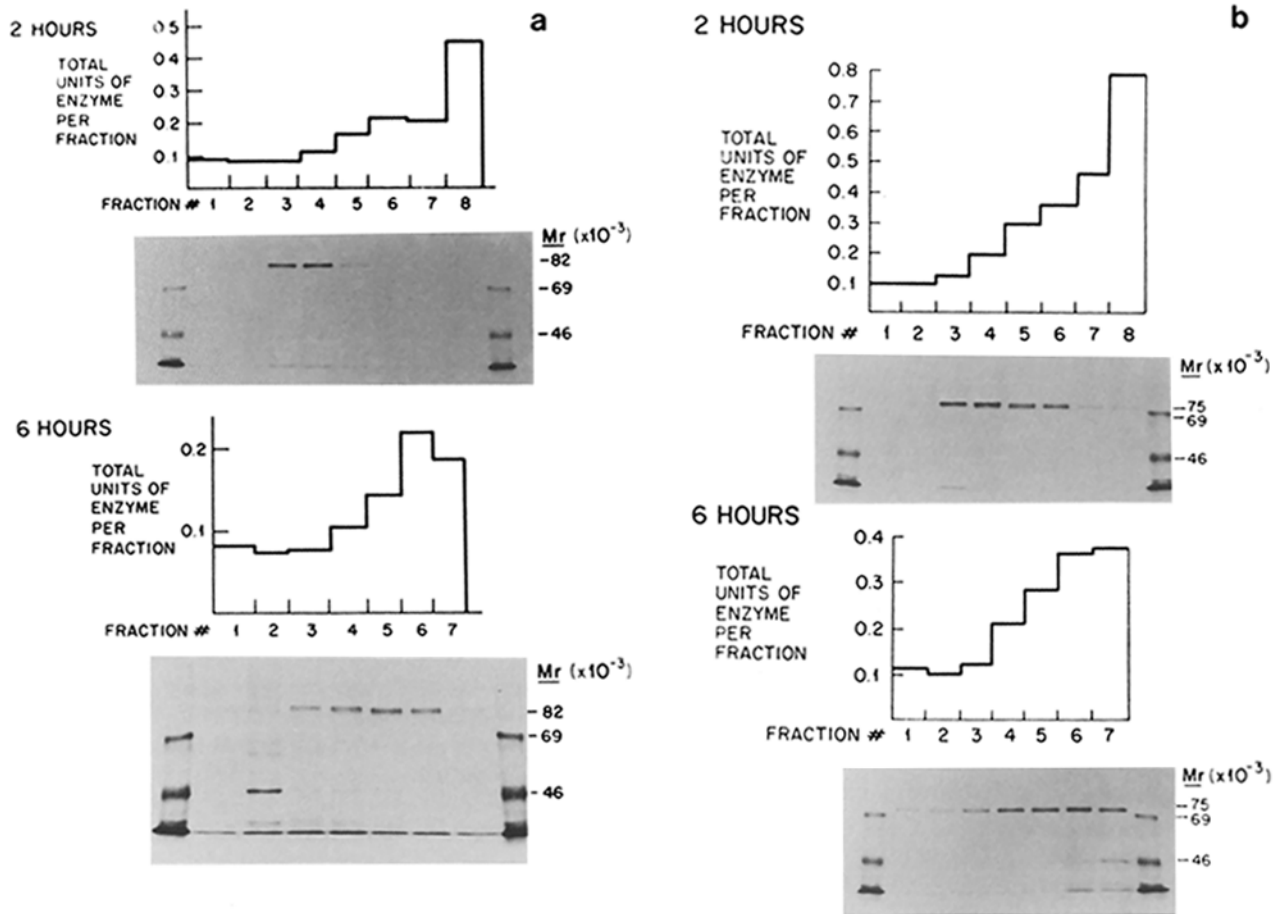
FIGURE 6   Subcellular distribution of newly synthesized β-glucuronidase and β-galactosidase in homogenates of untreated and ammonium chloride–treated macrophages. Cells were labeled for 1 h with 375 μCi [³⁵S]methionine/75-cm² flask in the presence of 25 mM ammonium chloride, then chased for 2 or 6 h with cold medium containing 25 mM ammonium chloride. At the end of each chase period the cells were harvested, homogenized, and centrifuged on a Percoll gradient as described (13). 1.0-ml fractions were collected from each gradient and assayed for β-galactosidase and β-glucuronidase activity, then each enzyme was purified by immunoprecipitations by monospecific antibodies bound to sepharose and electrophoresed on SDS gels. Fraction 1 corresponds to the least dense fraction obtained from the top of the gradient. The low molecular weight (43,000) component in fraction 2 of the 6-h β-galactosidase sample is not reproducible and therefore is a presumed contaminant. (a) β-Galactosidase. (b) β-Glucuronidase.

FIGURE 7 Specific secretion of the endo H–resistant form of galactosidase after ammonium chloride treatment. Macrophages were pulsed for 1 h with 125 μCi [³⁵S]methionine/25-cm² flask (untreated cells) or 375 μCi/75-cm² flask (cells treated with 50 mM ammonia), then chased for 6 h after ammonia was added (treated cells) or they were harvested immediately (untreated cells). β-Galactosidase was immunoprecipitated from the harvested cells and from the medium of the NH₄Cl-treated cells. Each immunoprecipitate was dissolved in endo H reaction buffer and divided into two aliquots. One aliquot was reacted with endo H and the second was used as a control without endo H. After reaction the samples were electrophoresed on SDS gels and prepared for autoradiography.
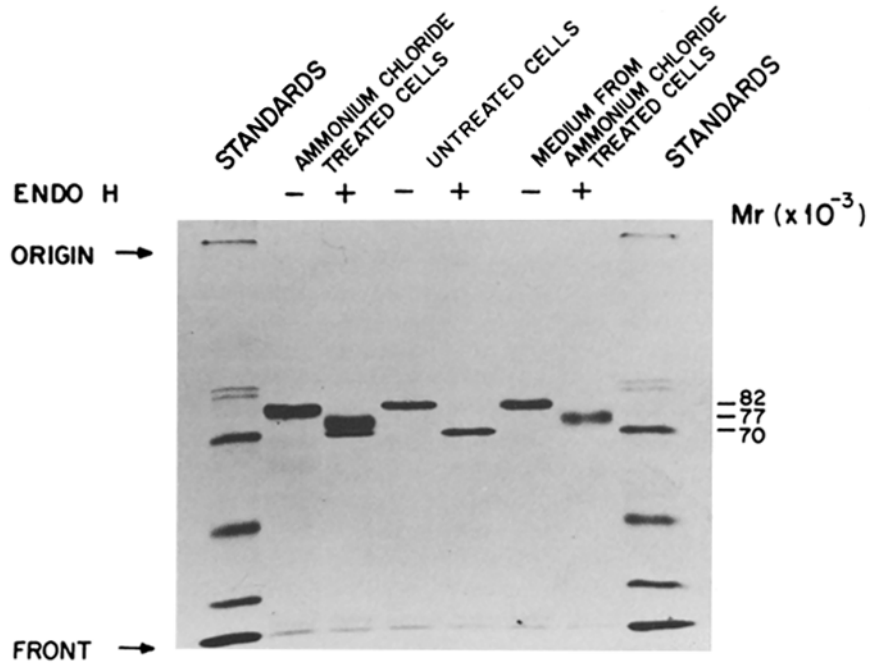
Secretion of Lysosomal Enzymes from Normal and Pale Ear Mice at Various Ammonia Concentrations

Enzyme	NH₄Cl	Percentage total enzyme activity secreted	
		Normal	Pale ear
	mM		
β-Glucuronidase	0	10	8
	5	45	15
	10	53	17
	50	51	18
β-Galactosidase	0	5	4
	5	36	7.7
	10	42	8.7
	50	51	12

Flasks of normal and pale ear mice were treated for 5 h at the indicated ammonia concentrations, and enzyme activity was measured in the cell-free media (secreted) and in cells.

In other experiments we determined that pale ear macrophages contain normal amounts of lysosomal enzymes and that ammonia treatment does not affect enzyme concentration. For example, β-galactosidase activity per flask of 10^7 macrophages was 1.5 and 1.8 U in untreated pale ear and normal macrophages, respectively, and remained essentially constant at 1.6 and 1.8 U per flask after 6 h of treatment with 50 mM ammonia. These data, along with the normal or above-normal levels of radiolabeled mature intracellular lysosomal enzymes found in untreated and treated pale ear macrophages (Fig. 8), indicate that the pale ear mutant has a true defect in secretion of mature lysosomal enzymes rather than an alteration in turnover of lysosomal enzyme within the cell or the medium after ammonia treatment. Also, pale ear macrophages process precursor forms of lysosomal enzymes to mature forms at the same rate and to completion (Fig. 9) as previously established in normal mice (12). At 2 h the percentage of mature β-galactosidase was 23.8% from pale ear and 26.7% from normal macrophage as analyzed by densitometry. Therefore, a defect in the maturation pathway does not explain the lowered secretion of mature form. Secretion of lysosomal enzyme activity from macrophages of untreated pale ear mice is depressed relative to secretion from untreated normal macrophages (previously shown to contain exclusively mature forms [12]), a result consistent with an effect of the pale ear gene on secretion of the contents of the lysosomal organelle.

DISCUSSION

The various subcellular routes of secretion of β-galactosidase, the relative contributions of the routes, and the effects of the pale ear gene on these pathways in normal and ammonia-treated macrophages are summarized in Figure 10.

Considerations of half-lives for precursor and mature forms of lysosomal enzymes in thioglycollate-stimulated macrophages (12) together with the observed secretion of up to 80% of total lysosomal enzymes in just 3 h in ammonia-treated macrophages indicated that mature lysosomal enzymes rather than precursors form the bulk of secreted activity. Indeed, the radiolabeling studies reported here directly show that low molecular weight mature lysosomal enzymes are secreted with parallel kinetics to secretion of bulk lysosomal enzyme activity. Also, we have presented evidence from Percoll gradient

fractionation that the bulk of secreted enzyme is derived from lysosomes (13). Together, therefore, the evidence strongly suggests that in stimulated mouse macrophages the bulk of the lysosomal enzyme secretion induced by ammonia is mature form previously contained in lysosomal organelles.

Riches et al. (8, 9) and Jessup et al. (10) observed that ammonia causes similar massive and rapid secretion of lysosomal enzyme activity from resident mouse peritoneal macrophages. Jessup et al. (31) observed that cycloheximide had little effect on ammonia-stimulated secretion from resident macrophages, which indicated that secreted enzymes were probably derived from preformed lysosomes rather than from newly synthesized enzymes. Our studies establish that most of the release is indeed low molecular weight mature enzyme from lysosomal organelles. Rapid secretion of bulk lysosomal enzyme may represent a normal function of macrophages, which are highly endocytic cells known to accumulate at sites of inflammation and which secrete their lysosomal enzyme content when challenged by a wide range of soluble and particulate stimuli (32–35).

A similarly high percentage release of precursor forms occurs upon ammonia treatment. A principal difference between ammonia-stimulated release of lysosomal enzymes in macrophages and fibroblasts, therefore, is that macrophages secrete both precursor and mature forms with the bulk of the release being mature enzyme whereas in fibroblasts ammonia has been reported to induce release of high molecular weight precursor but not low molecular weight mature forms (1). Furthermore, macrophages not treated with ammonia secrete only mature form (12), whereas untreated fibroblasts secrete only precursor (1). It has been reported (36) that chloroquine-treated fibroblasts secrete both precursor and mature lysosomal enzymes. However, direct evidence for the presence of both forms in secretions was not presented. Another major difference between macrophages and fibroblasts is that precursor enzyme forms are released much more slowly in fibroblasts (6, 31). That the bulk of the release in macrophages is in mature form also recommends caution in using ammonia-stimulated secretions as a source of newly synthesized enzyme in uncharacterized cells.

Imort et al. (20) found, in contrast to results with mouse macrophages, that ammonia causes release only of precursor lysosomal enzymes in human monocyte-derived macrophages, although zymosan causes release of both precursor and mature forms. Some of the above differences may therefore reflect species rather than cell type differences.

It is apparent that precursor and mature forms are secreted by quite different molecular and subcellular mechanisms after ammonia treatment. That normal precursor enzyme secretion occurred in macrophages of pale ear mice, which have lowered secretion of mature enzyme, is compelling evidence that precursor enzyme is secreted via a nonlysosomal route. A likely possibility is that precursors in ammonia-treated cells are rerouted through the Golgi apparatus along the same route taken by secretory proteins (37). Previous studies (13) have established that the low density region of the Percoll gradients shown in these studies to be the principal location of precursor enzyme contain elements of the Golgi apparatus, plasma membrane, and endoplasmic reticulum. Also, the fact that secreted β-galactosidase precursor is exclusively of the form relatively resistant to endo H digestion is further evidence that this precursor, at least, has traversed the Golgi apparatus before secretion. This finding is similar to the
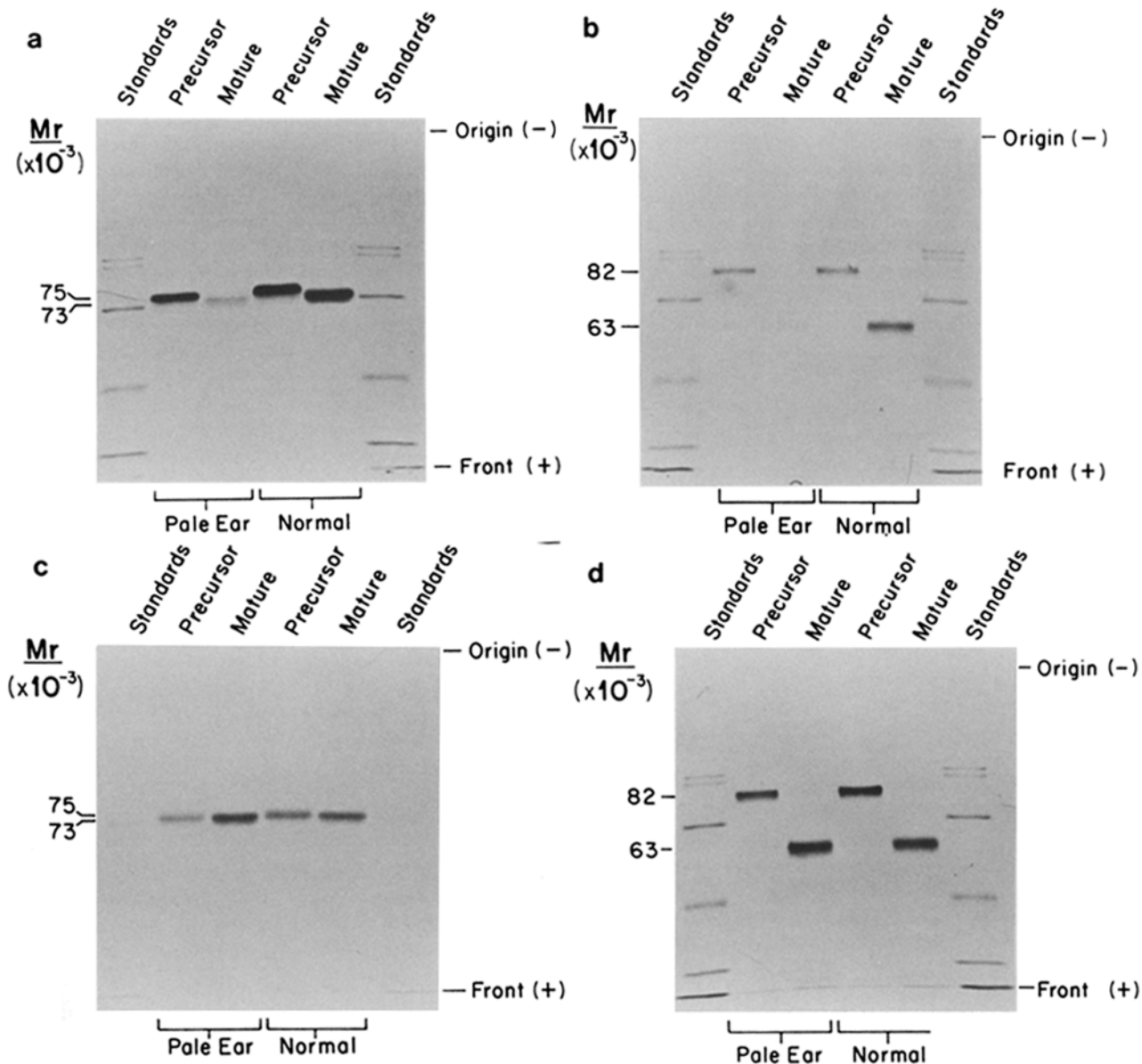
FIGURE 8 Secretion of precursor and mature forms of lysosomal enzymes from ammonia-treated macrophages of normal and pale ear mice. Macrophages were isolated from C57BL/6J (+/+) and pale ear (ep/ep) mice and cultured in vitro. For precursor studies the cells were labeled for 15 min with 125 μCi [³⁵S]methionine/25-cm² flask and then chased for 6 h, both in the presence of 50 mM ammonium chloride, and then the cells and medium were harvested. For studies on mature enzyme, the cells were labeled for 15 min, then chased with cold medium for 24 h in the absence of added ammonia. The medium was then replaced with medium containing 50 mM ammonium chloride and incubated for 3 h more. The cells and medium were then harvested, and β-glucuronidase and β-galactosidase were purified from each fraction by sequential immunoprecipitation with monospecific antibodies and electrophoresed on SDS gels followed by autoradiography. (a) β-Glucuronidase isolated from the medium. (b) β-Galactosidase isolated from the medium. (c) Cellular β-glucuronidase. (d) Cellular β-galactosidase.

observation of additional processing of secreted lysosomal enzyme oligosaccharides to complex forms observed in fibroblasts from patients with I-cell disease (38).

The increased flux of lysosomal enzyme precursors through the Golgi apparatus with attendant oligosaccharide modification while enroute to secretion in ammonia-treated fibroblasts is thought to result from decreased availability of unoccupied receptors, which normally transport enzyme to lysosomes (1, 6). A similar explanation may hold for secretion of precursors from treated macrophages. In several respects the different intracellular secretory routes of precursor and mature macrophage lysosomal enzymes resemble the two

intracellular routes followed by secreted precursor and mature adrenocorticotropic hormone (39).

Further experiments are required to determine whether the interesting difference in lag time for secretion of newly synthesized β-glucuronidase and β-galactosidase precursors is due to separation in routes of secretion or to altered rates of interaction with intracellular molecules that regulate secretion. It is not due to an intrinsic defect in secretion of β-galactosidase precursor since longer incubations (6 h) result in equal secretion of the two precursors. It is also not due simply to a larger intracellular pool size of β-galactosidase precursor, since this and previous (12, 13) studies have dem-

onstrated that β-glucuronidase precursor is synthesized at a greater rate than β-galactosidase precursor, and, in contrast to the differential secretory rates, the precursors are transferred from the prelysosomal to the lysosomal intracellular pools with similar kinetics in both normal and ammonia-treated cells. Others have found that monensin (40) and cyanate (41) stimulate the secretion of the precursor of cathepsin D but not of β-hexosaminidase from fibroblasts. Also, similar differential intracellular transport rates have been noted for membrane (42) and secretory (43) proteins.

There is a pronounced differential sensitivity to ammonia processing of the β-glucuronidase and β-galactosidase precursors. The Percoll gradient result suggests that the β-glucuron-

idase maturation that takes place in treated cells probably occurs either in lysosomes or just before delivery to lysosomes, a conclusion consistent with results in untreated macrophages (13). The differential sensitivity of cleavage of precursors of β-glucuronidase and β-galactosidase suggests either that different cleavages occur (44) or that these enzymes are processed by different molecular or subcellular mechanisms.

The molecular mechanism of reduced secretion from macrophages of pale ear mice is unknown though additional information is accumulating about the organellar defects in these and other mouse pigment mutants. The pale ear gene has been backcrossed onto the C57BL/6J inbred strain 20 times to form a congenic strain that is essentially identical in genotype to the C57BL/6J strain except for the chromosome 19 site of the pale ear gene. One can therefore be quite confident that any differences observed between pale ear and normal mice are regulated by the chromosomal site surrounding the pale ear gene rather than by different background genes. Recent studies in this laboratory and others have established that the pale ear mutant and several other mouse pigment mutants can be thought of as "organellar" mutants in that they affect the structure or processing of several subcellular organelles. These mutants have altered structure and/or transfer of melanosomes from melanocytes to keratinocytes (45), abnormal structure and/or secretion of lysosomes of several cell types (25–30, 46), and a marked deficiency of platelet dense granules (29, 30).

The experiments reported in this article suggest that the pale ear gene is affecting either the mature lysosomal organelle of macrophages or the interaction of this organelle with the secretory apparatus, inhibiting its secretion by as yet undefined mechanisms. To our knowledge this is the first description of a mutant defective in secretion of any type of macrophage protein. Many secretion mutants have been described in yeast (47) and slime mold (48). Macrophages from pale ear mice could therefore be a useful tool in examining the relatively little understood process of exocytosis of the contents of mature lysosomes.

TABLE II

Secretion of Precursor and Mature Lysosomal Enzymes from Treated Normal and Pale Ear Macrophages

	Normal	Pale Ear
β-Glucuronidase		
Precursor		
Total cpm	8,440	6,050
% Secreted	40.9	41.7
Mature		
Total cpm	8,440	7,240
% Secreted	32.9	9.1
β-Galactosidase		
Precursor		
Total cpm	2,360	1,780
% Secreted	28.8	32.9
Mature		
Total cpm	3,240	2,400
% Secreted	42.7	8.5

Radiolabeling of precursor and mature lysosomal enzymes forms and stimulation of secretion by treatment with ammonia were performed as described in Fig. 8. Counts per minute in total trichloroacetic acid precipitable protein were 4.67×10^6 for normal and 4.75×10^6 for pale ear macrophages under precursor labeling conditions, and 4.77×10^6 for normal and 2.78×10^6 for pale ear macrophages under mature labeling conditions.

FIGURE 9 Kinetics of processing of lysosomal enzyme precursors in normal and pale ear macrophages. Macrophages were labeled for 15 min with 125 μCi [^{35}S]methionine/25-cm^2 flask and then chased for 2 and 6 h under normal unlabeled culture conditions. The cells were harvested, and β-galactosidase was purified by immunoprecipitation and electrophoresed on SDS gels followed by autoradiography.
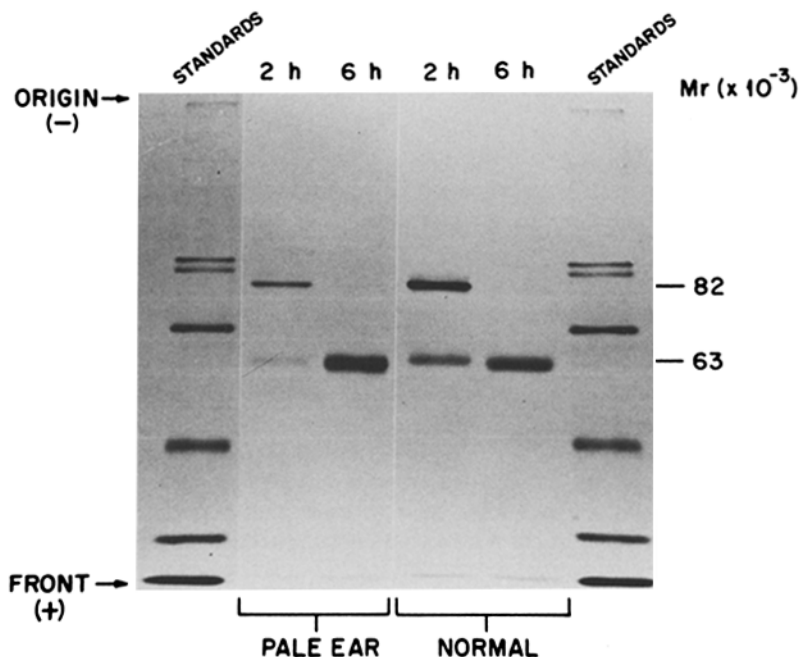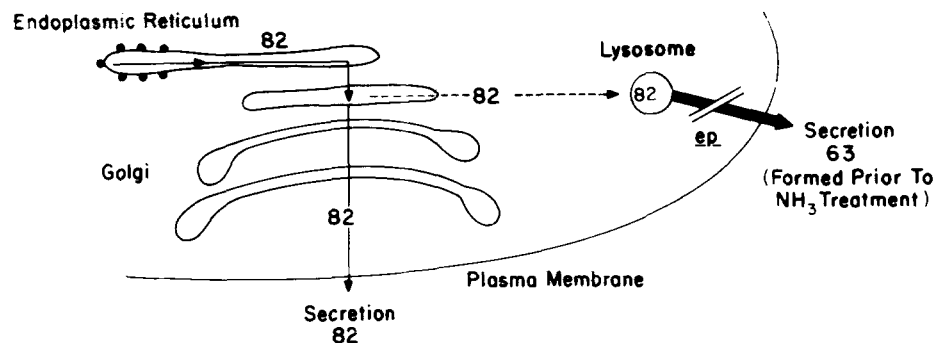
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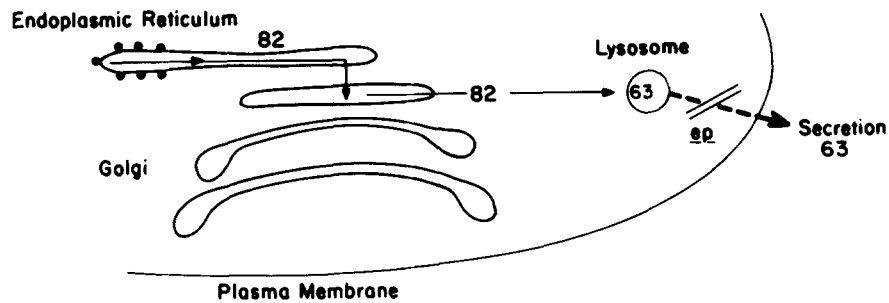
B

Figure 10 Schematic representation of processing and secretion of β-galactosidase in untreated (A) and ammonia-treated (B) macrophages. The 82,000-mol-wt precursor in untreated cells is rapidly transferred to lysosomes and is cleaved to the 63,000-mol-wt mature form either immediately before or immediately after reaching the lysosome (12, 13). There is a small but significant secretion of mature form, probably by exocytosis of lysosomes, and this process is regulated by the pale ear (ep) gene product. No detectable precursor secretion occurs. Although we have depicted the normal intracellular routing of lysosomes as involving portions of the Golgi apparatus, other intracellular pathways are possible (discussed in reference 13). In ammonia-treated cells, maturation of the 82,000-mol-wt precursor is inhibited, as is transport to lysosomes. Approximately one-half of the precursor is secreted through the Golgi apparatus, where it acquires complex oligosaccharides. A portion of the remaining cellular precursor is slowly transported to lysosomes. Massive secretion of lysosomal enzyme contents is a hallmark of treated macrophages, and again the exocytosis of mature lysosomes is under the control of the ep gene. Synthesis, processing, and secretion of β-glucuronidase follows pathways parallel to those indicated for β-galactosidase. However, in untreated cells, mature β-glucuronidase is secreted at twice the rate of β-galactosidase. Also, in treated cells there is a significantly shorter lag for secretion of β-glucuronidase precursor and maturation of the β-glucuronidase precursor is relatively resistant to ammonia.

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