S. J., Ed., Academic Press, New York, N.Y., pp 445–593.
Wee, T. G., and Frey, P. A. (1973), *J. Biol. Chem. 248*, 33–40.
Wilson, D. G., and Hogness, D. S. (1964), *J. Biol. Chem. 239*, 2469–2481.
Wilson, D. B., and Hogness, D. S. (1969), *J. Biol. Chem. 244*, 2132.
Wong, S. S., and Frey, P. A. (1977), *Biochemistry 16*, 298–305.

Extracellular Hydrolases of the Lung†

Gary F. R. Hook

With the Technical Assistance of Linda B. Gilmore

ABSTRACT: A pool of acid hydrolases exists within the acellular lining material of the alveoli and distal airways of the lungs. These extracellular hydrolases, obtained using pulmonary lavage procedures, appear to be of a selected variety insofar as some hydrolases (β-*N*-acetylglucosaminidase and α-mannosidase) are highly active while others (β-glucuronidase and arylsulfatase) are barely detectable. The origins of these hydrolases were investigated. Neither leakage of serum nor cell damage can account for the presence of the extracellular hydrolases in lavage effluents. Electrophoretic mobilities on acrylamide gels indicate that the extracellular hydrolases generally differ from those found in serum. Cytoplasmic soluble enzymes such as lactate dehydrogenase were used to monitor cell damage and show that the extracellular hydrolases did not originate from cell leakage during the lavage procedure. Hydrolases similar to those found extracellularly are associated with highly purified lysosome-free lamellar bodies isolated from homogenates of lung. The extracellular hydrolases are probably secreted by the type 2 cells of the pulmonary alveolar epithelium during their secretion of lamellar bodies.

The distal airways of the lungs, that is, the bronchioles and alveoli, are lined with an acellular layer of material whose presence is essential for the maintenance of normal pulmonary function. Although this acellular lining has been the subject of numerous investigations, the composition of the lining has not been elucidated completely and many of its properties have been recognized only recently.

The lipid components have been investigated extensively, especially those surface-active phospholipids such as dipalmitoyllecithin which play a major role in the stabilizing influence of the acellular lining in the distal regions of the lungs (Macklem et al., 1970; Pattle, 1958); however, the composition of the lining is not confined to phospholipids and many other components, particularly proteins, have been detected (Bhattacharyya et al., 1975; Bignon et al., 1975, 1976; Hand and Cantey, 1974; Reynolds and Newball, 1974).

The acellular lining as an enzymatically active medium has not been considered previously. In this report evidence is presented for the existence of selected lysosomal hydrolases within the acellular lining of the alveoli and distal airways of the lungs.

Materials and Methods

Materials. Adult male rabbits of the New Zealand strain (Dutchland Laboratory Animals Inc., Denver, Pa.) weighing from 2 to 2.5 kg were used throughout. Rabbits were allowed free access to food and water.

Chemicals used are listed with their sources in parentheses as follows: disodium *p*-nitrophenyl phosphate, *p*-nitrophenyl acetate, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl α-D-mannoside, *p*-nitrophenyl palmitate, *p*-nitrophenyl β-D-galactopyranoside, dipotassium *p*-nitrocatechol sulfate, α-ketoglutarate, DL-aspartate, NADH, malic dehydrogenase (Sigma Chemical Co., St. Louis, Mo.); sodium pyruvate, phenolphthalein glucuronide sodium salt (Calbiochem, San Diego, Calif.); Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.); acrylamide, *N,N'*-methylenebisacrylamide (Eastman Kodak Co., Rochester, N.Y.); bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.).

Pulmonary Lavage Procedure. Rabbits were killed by injection of 3–4 mL of sodium pentobarbital solution (50 mg/mL) into the marginal ear vein. The trachea was exposed and clamped shut immediately after inflation of the lungs by caudad pressure on the diaphragm. The trachea and lungs were removed intact and carefully dissected free of other tissues. The external surfaces of the lungs were rinsed free of blood using Hanks' solution (Hanks and Wallace, 1949). The trachea was cut approximately 1 cm below the clamp, gently closed below the bifurcation, and tracheal airway surfaces rinsed with Hanks' solution. A plastic tube was then inserted into the trachea and the lungs were made to respire, using minimal positive air pressure via the tube, until any signs of atelectasis had gone. The lungs and the trachea were then filled with ice cold Hanks' solution. No attempts were made to force the lavage medium into the lungs. The lungs were filled to the top of the trachea which was about 5 cm long. The external surfaces of the lung were very gently massaged for approximately 30 s to assist dispersion of the Hanks solution. The lungs were then inverted and the lavage fluid allowed to drain into the collecting vessel. The lavage procedure was repeated as many times as required by experimental design. Although the volumes of lavage effluents varied among different lungs, consecutive lavages were very similar (standard deviation amounted to less than 12%). The trachea was wrapped in several layers of absorbent gauze prior to drainage to avoid

† From the Laboratory of Pharmacology, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, North Carolina 27709. *Received September 8, 1977.*

contamination of the lavage effluent with blood from the external surfaces of the lungs. Lavage effluents were not used if erythrocytes were detectable in the medium. Lavage effluents from rabbits were rendered cell-free by centrifugation at $580g_{av}$ for 10 min. Prior to enzyme analyses, Triton X-100 was added to the cell-free lavage effluents (final concentration of Triton X-100 was 0.1%). Triton X-100 was added for two reasons: first, to allow hydrolase activities in different lung fractions to be compared under the same media conditions and, second, to disperse hydrolases from noncellular particulate components present in the lavage effluents [approximately 5– of the total extracellular acid phosphatase is associated with sedimentable membranous materials which can be dispersed using Triton X-100 (G. E. R. Hook, unpublished observations)].

Alveolar Macrophages. Cells present in lavage effluents from rabbit lungs were sedimented at $580g_{av}$ for 10 min. The pelleted cells were washed once in Hanks' solution. Cell suspensions consisted of at least 90% alveolar macrophages and generally less than 0.5% polymorphonuclear leukocytes as determined by light microscopy with hematoxylin and eosin staining. Cells were suspended in Hanks' medium and disrupted by sonication at 80 W (Sonifier Cell Disruptor Model W185, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) for three 10-s intervals with ice cooling. The suspension was cooled for 2 min between sonications. Sonication under these conditions did not appear to affect enzyme activities. Cell disruption was complete as shown by microscopic examination. Triton X-100 was added to a final concentration of 0.1% to ensure complete rupture of lysosomes. The cell homogenates were then centrifuged at 178 $000g_{av}$ for 60 min. Enzyme analyses were carried out on the 178 $000g_{av}$ supernatant.

Subcellular Fractions. For the determination of total enzyme activities, lungs without trachea were passed through a tissue press (Arbor Tissue Press, Model No. 142, Harvard Apparatus Co., Inc., Dover, Mass.) and then homogenized in distilled water (5 mL/g of lung) using a Polytron Homogenizer, Model PT 10-35 (Brinkman Instruments, Westbury, N.Y.), at full power for 15-s intervals. The temperature was maintained below 10 °C and the total homogenization time was 2 min. Lungs were also homogenized in Hanks' solution but the release of lysosomal enzymes was found to be consistently more complete when distilled water was used as the medium; however, total activities were similar in both media. The homogenate was centrifuged at 96 $000g_{av}$ for 30 min. The supernatant was withdrawn and the pellet resuspended in distilled water to the original homogenate volume. The suspension was then rehomogenized and recentrifuged as described above. The pellet was again suspended in distilled water and again homogenized and centrifuged as described for the original lungs. The 96 $000g_{av}$ supernatants were not combined. The final pellet was suspended in distilled water. The total enzyme levels in the lungs were estimated from the sum of the activities in the supernatants and final pellet suspension. Each fraction was mixed with an equal volume of double strength Hanks' solution containing 0.2% Triton X-100 so that the medium was similar to that containing the lavage effluents.

The soluble phases of lungs which had been lavaged were prepared in such a way as to minimize enzyme leakage from lysosomes (Hook et al., 1972). Lavaged lungs (without trachea) were homogenized in 0.25 M sucrose (3.5 mL/g of lung) using a Potter-Elvehjem homogenizer (six passes of the Teflon pestle). The homogenate was centrifuged at $2300g_{av}$ for 20 min (Sorvall RC2-B; SS-34 rotor). The pellet was discarded and the supernatant centrifuged at 178 $000g_{av}$ (Beckman L3-50; 60 Ti rotor) for 30 min. This procedure resulted in the release of at least 90% of the cytoplasmic soluble protein and associated soluble marker lactate dehydrogenase. Appropriate quantities of Hanks' solution and Triton X-100 were added to the cytosol preparation prior to the assay of hydrolases so that the medium was similar to that of the lavage effluents.

Preparation of Lamellar Bodies and Lysosomes: Analysis by Isopycnic Density Gradient Centrifugation. Lysosome-rich fractions were prepared from trachea-free lungs. The lungs were passed through a tissue press and then homogenized in 0.25 M sucrose (4 mL/g of lung) using a Potter-Elvehjem homogenizer consisting of a Teflon pestle with a smooth glass grinding chamber (clearance 0.006–0.009 in.). Six passes of the motor-driven pestle (2500 rpm) were used to disrupt the tissue. The homogenate was centrifuged at $3300g_{av}$ for 10 min (Sorvall RC-2B centrifuge, SS-34 rotor). The pellet was discarded and the supernatant centrifuged at 10 $000g_{av}$ for 20 min (Sorvall RC-2B, SS-34 rotor). The pellet was resuspended in Hanks' solution.

Lamellar bodies were prepared from lungs using the procedure in DiAugustine (1974) and finally suspended in Hanks' solution.

The lamellar body- and lysosome-rich fractions, each suspended in a volume of 4.0 mL of Hanks' solution, were layered over sucrose density gradients which had also been prepared in Hanks' solution. The inclusion of Hanks' solution in the gradients was necessary for the preservation of the lamellar bodies. Lysosomes were quite stable in this medium as well. The gradients were centrifuged at 81 $500g_{av}$ (Beckman SW 27 rotor) for 16 h. Gradients were fractionated by injecting high density sucrose solution (density 1.19) through the bottom of the tubes; 0.8-mL fractions were collected. Densities were determined from the refractive index.

Enzyme, Protein, and Phospholipid Analyses. Although fractions of the lungs were prepared under conditions which differed but which appeared optimum for each particular fraction, where comparisons between fractions were required, these differences were minimized by appropriate adjustments to each media. When comparisons between fractions of the lungs were made, hydrolase activities were generally measured in the presence of 0.01% Triton X-100, 0.025 M sucrose, and 0.1 × strength Hanks' medium. These concentrations allowed the hydrolase activities in different fractions to be estimated under identical conditions; also, at these concentrations neither Triton X-100, sucrose nor Hanks' appeared to affect the hydrolases measured. Substrates used are indicated in parentheses: nonspecific esterase (*p*-nitrophenyl acetate) (Vatter et al., 1968), β-glucuronidase (EC 3.2.1.31) (phenolphthalein glucuronide) (Gianetto and DeDuve, 1955), acid phosphatase (EC 3.1.3.2) (*p*-nitrophenyl phosphate) (Turnbull and Neil, 1969), arylsulfatase (EC 3.1.6.1) (2-hydroxy-5-nitrophenyl sulfate) (Dodgson et al., 1955), β-galactosidase (EC 3.2.1.23) (*p*-nitrophenyl β-D-galactopyranoside) (Levvy and McAllen, 1963), β-*N*-acetylglucosaminidase (EC 3.2.1.53) (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) (Sellinger et al., 1960), α-mannosidase (EC 3.2.1.24) (*p*-nitrophenyl α-D-mannoside) (Conchie and Hay, 1959), fatty acid esterase (*p*-nitrophenyl palmitate) (Mahadevan and Tappel, 1968), alkaline phosphatase (EC 3.1.3.1) (Reasor et al., 1978), lactate dehydrogenase (EC 1.1.1.27) (pyruvate:NADH) (Schwartz and Bodansky, 1966), glutamic oxalacetic transaminase (EC 2.6.1.1) (DL-aspartate) (Bergmeyer, 1965).

Protein was determined using the method of Lowry et al. (1951). Phospholipids were extracted using the procedure of Folch et al. (1957) and measured using the method of Shin (1962).

Electrophoresis. Hydrolase-containing samples were treated

TABLE I: Extracellular and Total Hydrolases of the Rabbit Lung.

	Total lung[a]	Cell-free lavage effluent[b]	%[c]
α-Mannosidase	166.8 ± 31.1[d,e]	24.3 ± 4.6	14.6
β-*N*-Acetylglucosaminidase	832.8 ± 100.9	69.0 ± 5.5	8.3
β-Galactosidase	82.4 ± 10.7	2.7 ± 0.5	3.3
Acid phosphatase	528.8 ± 25.7	6.6 ± 2.5	1.3
Fatty acid esterase	45.8 ± 7.7	0.54 ± 0.41	1.2
β-Glucuronidase	9.81 ± 0.30	0.07 ± 0.05	0.7
Arylsulfatase	76.1 ± 14.9	0.16 ± 0.17	0.2
Alkaline phosphatase	402.9 ± 44.8	11.3 ± 4.0	2.8

[a] For the estimation of total hydrolase activities, lungs were processed as described under Materials and Methods. [b] Lungs were lavaged six times with Hanks' medium. The lavage effluent was rendered cell-free by centrifugation at $580g_{av}$ for 10 min. [c] Percent of the total lung activity found in cell-free lavage effluents. [d] Hydrolase activities expressed as μmol of substrate hydrolyzed per 30 min per total lung or cell-free lavage effluent. [e] Mean ± SD. Four individual rabbits were used for the determination of total hydrolase activities in the lungs and a different four rabbits were used for the estimation of hydrolase activities in cell-free lavage effluents.

TABLE II: Cytoplasmic Soluble Enzymes in Pulmonary Lavage Effluents.[a]

	Cell-free lavage effluent[b]	Lungs total act.[b]	% extracellular
Glutamic oxalacetic transaminase	0.33 ± 0.16 (22)[c]	54.27 ± 5.07 (4)	0.61
Lactate dehydrogenase	0.87 ± 1.24 (26)	191.59 ± 24.74 (4)	0.45

[a] Lungs were lavaged six times and cells were removed by centrifugation. The total enzyme content of lungs was determined as described under Materials and Methods. [b] μmol of NADH oxidized/min. [c] Mean ± SD (*N*), where *N* = number of rabbits.

with 0.1% Triton X-100 prior to electrophoresis on acrylamide gel rods containing 4.3% acrylamide, 0.1% bisacrylamide (Green et al., 1972) and 0.1% Triton X-100. Resolution of the enzymes in the gel was improved by the inclusion of Triton X-100. Following electrophoresis the gel rods were sliced into 2-mm sections and incubated with the appropriate substrate at 37 °C. Homogenizing the gel slice prior to incubation with substrate only marginally increased the enzyme activities.

Electron Microscopy. For electron microscopy of the two major particulate components present in lamellar body preparations, only the three central fractions of each peak from the density gradients were used. The fractions were combined and diluted to twice their volume with 0.25 M sucrose in Hanks' solution and then centrifuged at $30\,000g_{av}$ for 20 min. The supernatants were removed and discarded. The pellets were fixed in 2% glutaraldehyde–1% paraformaldehyde buffered with 0.1 M phosphate, pH 7.4, for 4 h. Postfixation with osmium tetroxide, treatment with uranyl acetate, and dehydration in acetone were carried out according to the procedure of Williams (1977). The pellets were embedded in Epon (Luft, 1961). Ultrathin sections were cut on a Porter-Blum Ultramicrotome MT-1 using a duPont (E. I. duPont de Nemours and Co., Wilmington, Del.) diamond knife. The sections were stained with 5% uranyl acetate in ethanol (Watson, 1958) for 10 min and then with lead citrate (Reynolds, 1963) for 6 min and were examined in a Philips 300 electron microscope.

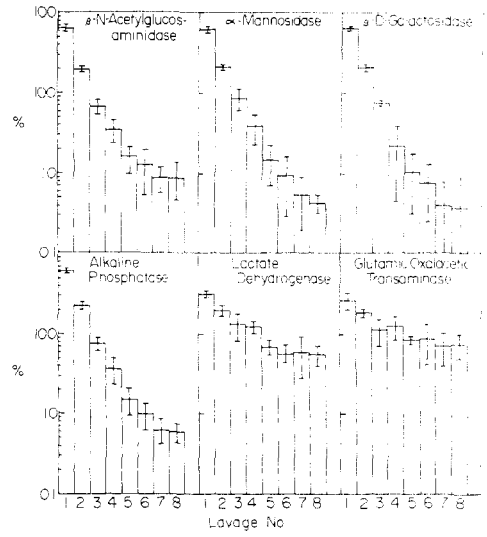

FIGURE 1: Removal of extracellular enzymes from rabbit lungs by consecutive lavages. Lungs were lavaged as described under Materials and Methods. For a given lung, the volumes of consecutive lavage effluents were similar. Results are from four rabbits with vertical bars representing the mean quantity of enzyme removed by each lavage expressed as a percentage of the total enzyme removed by all eight consecutive lavages. Standard deviations are indicated.

Results

Extracellular Hydrolases. The total activities of alkaline phosphatase and several acid hydrolases present in homogenates of rabbit lungs and cell-free pulmonary lavage effluents are shown in Table I. Those hydrolases, which are the most active in homogenates of lungs, tend to be the most active in cell-free pulmonary lavage effluents, although the orders of the activities are not exactly the same. Substantial proportions of the total α-mannosidase and β-*N*-acetylglucosaminidase associated with the lungs (14.6 and 8.3%, respectively) appear to exist in a cell-free state. Arylsulfatase, β-glucuronidase, and fatty acid esterase, as indicated by their low values and large standard deviations, are often barely detectable in cell-free lavage effluents.

The presence of the cytoplasmic soluble enzymes glutamic oxalacetic transaminase and lactate dehydrogenase in the cell-free lavage effluents (Table II) indicates that cells could have been damaged prior to or during the lavage procedure. However, the quantity of each of these enzymes in the cell-free lavage effluent amounts to less than 1% of the total lung content; consequently, it seems unlikely that the presence in lavage effluents of most of the extracellular hydrolases listed in Table I is due to their leakage from the cytoplasm of damaged cells. In addition, neither glutamic oxalacetic transaminase nor lactate dehydrogenase (nor any of the hydrolases listed in Table I) were released from alveolar macrophages following their removal from the lungs, when maintained in suspension for periods of up to 5.5 h or when subjected to centrifugal forces of up to $27\,000g_{av}$ for 30 min (data not shown).

The removal of a number of enzymes from the lungs with consecutive lavages is illustrated in Figure 1. For purposes of comparison, the quantity of enzyme removed with each lavage is expressed as a percentage of the total removed by all eight lavages. Eight lavages were chosen since hydrolase activities in further lavage effluents were barely detectable (extrapolation of the data using the models discussed below indicates that eight lavages remove approximately 95% of the total theoretically available extracellular hydrolases from the airways of the lungs but only about 60% of the theoretically available

lactate dehydrogenase and glutamic oxalacetic transaminase). As indicated by the standard deviations of the data shown in Figure 1, the lavage procedure appears to be highly reproducible when carried out by the described procedure (see Materials and Methods). Although the lavage number is actually a discrete rather than continuous variable, it is nonetheless possible to fit curves to the data shown in Figure 1. When this is done the shapes of the curves indicate that the enzymes are being removed from compartments within the lungs and that the removal process involves the partitioning of the enzymes between their lung compartments and the lavage medium. The derived equations simplify statistical comparisons between the enzymes and permit limited examination of those pulmonary compartments from which the enzymes present in cell-free lavage effluents originate.

Assuming a first-order process for the partitioning of each enzyme between its pulmonary compartment and the lavage fluid, the removal of an enzyme from its pulmonary compartment by consecutive lavages should be described by an equation of the form $y = a\exp(-bx)$, where y is the quantity of enzyme removed from its lung compartment by the xth lavage ($x = 1, 2, 3 \ldots$) and a and b are constants. However, for the hydrolases significantly better fits were obtained using a two-compartment model, where $y = a\exp(-bx) + c\exp(-dx)$ (a, b, c, and d are constants and x and y as previously defined).[1] If a two-compartment model is assumed, then the differences among the enzymes β-N-acetylglucosaminidase, α-mannosidase, β-galactosidase, and alkaline phosphatase are not statistically significant. That is, estimation of the parameters a, b, c, and d for each of the enzymes does not provide a significant improvement over a single two-compartment model with $a = 182.3 \pm 27.0$, $b = 1.08 \pm 0.08$, $c = 0.92 \pm 0.92$, and $d = 0.085 \pm 0.130$. Consequently, it would appear that the hydrolases β-N-acetylglucosaminidase, α-mannosidase, β-galactosidase, and alkaline phosphatase are all lavaged from the same two pulmonary compartments. However, hydrolase contributions from the two compartments are unequal, approximately 94% being derived from the major and only 6% from the minor compartment for a total of eight consecutive lavages.

Similarly, the equations derived for lactate dehydrogenase and glutamic oxalacetic transaminase do not differ significantly from a single two-compartment model with estimated parameters $a = 35.4 \pm 8.3$, $b = 0.66 \pm 0.38$, $c = 10.88 \pm 13.0$, and $d = 0.079 \pm 0.151$, although the equations do differ significantly from those of the hydrolases. Here it should be noted that a two-compartment model for lactate dehydrogenase and glutamic oxalacetic transaminase gave only marginal improvement over a single-compartment model. It would appear, then, that lactate dehydrogenase and glutamic oxalacetic transaminase are contributed to the lavage medium from the same source(s) but that source(s) is not the same as that from which the hydrolases are derived.

Some indications may be obtained from Figure 2 as to the nature of the pulmonary compartments from which the extracellular enzymes are derived. The linear relationships between the quantities of β-N-acetylglucosaminidase, alkaline phosphatase, and phospholipid lavaged from the lungs indicate that the two hydrolases and phospholipid share a common

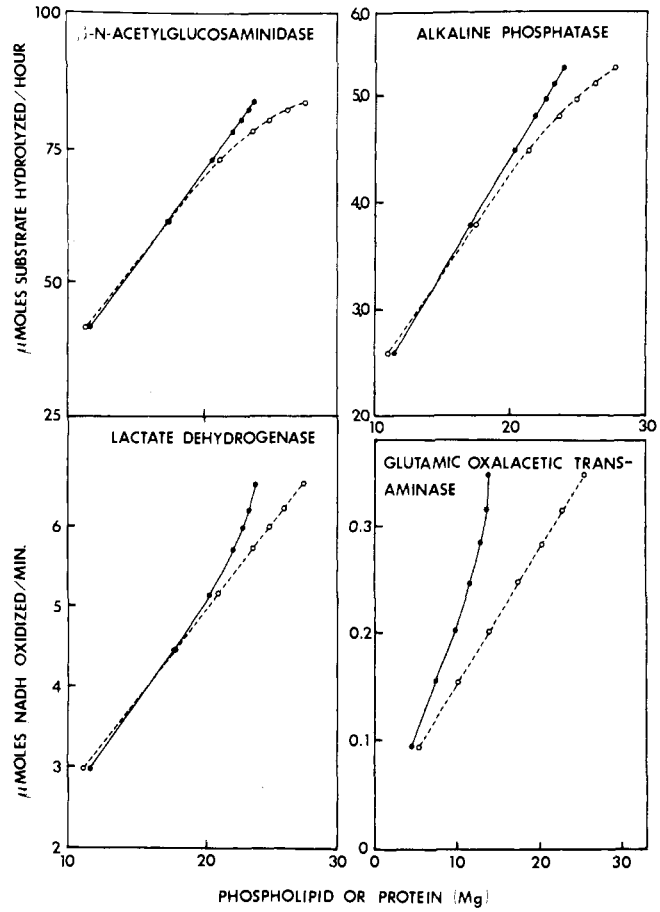

FIGURE 2: Relationships between extracellular enzymes, phospholipid, and protein present in pulmonary lavage effluents from the rabbit. Lungs were lavaged and cells removed as described under Materials and Methods. Protein, phospholipids, and enzyme activities were determined in cell-free lavage effluents and plotted as total quantities removed from the lungs. β-N-acetylglucosaminidase, alkaline phosphatase, and lactate dehydrogenase were obtained from the same rabbit: glutamic oxalacetic transaminase is from another rabbit. (●—●) phospholipid; (O- -O) protein.

compartment. Such a relationship is also seen between phospholipid and other hydrolases such as α-mannosidase and β-galactosidase (data not shown). Since the major phospholipids of cell-free lavage effluents arise from the acellular lining of the pulmonary distal airways, it would appear likely that the extracellular hydrolases also originate from the same acellular lining.

Linear relationships between extracellular hydrolases and protein present in lavage effluents, although present during early stages of the lavage procedure, are not maintained (Figure 2). However, the removal of the cytoplasmic soluble enzymes lactate dehydrogenase and glutamic oxalacetic transaminase from the lungs is directly proportional to the quantity of protein present in the cell-free lavage effluents. Thus, lactate dehydrogenase, glutamic oxalacetic transaminase and protein may share a common source. Since cytoplasmic soluble enzymes in cell-free lavage effluents may arise from cell leakage during and/or before the lavage procedure, major protein constituents may also originate from damaged cells.

The Origins of Extracellular Hydrolases. All of the enzyme activities present in cell-free lavage effluents so far considered are also detectable in blood plasma. Since the specific activities (on a protein basis) of enzymes present in cell-free lavage effluents are generally two orders of magnitude higher than the corresponding activities found in plasma (Table III), it seems unlikely that the lavage effluent enzymes could be accounted

[1] One- [i.e., $y = a\exp(-bx)$] and two-compartment [i.e., $y = a\exp(-bx) + c\exp(-dx)$] models were fitted to the data by estimating a, b, c, and d so as to minimize $R = \Sigma_{i=1}^{N}[\ln(y_i) - \ln(\hat{y}_i)]^2$, where $\hat{y}_i$ is obtained by replacing the unknown parameters by their least-squares estimates. For the hydrolases, the two-compartment model provided a significant reduction in R relative to a one-compartment model.

TABLE III: Specific Activity of Some Enzymes Found in Blood Plasma and Pulmonary Lavage Effluents.[a]

	Plasma	First lavage effluent
Arylsulfatase[b]	0.918 ± 0.150 (4)[d]	206, 245
β-*N*-Acetylglucosaminidase[b]	16.3 ± 5.1 (4)	5101 ± 1591 (5)
Acid phosphatase[b]	3.75 ± 0.62 (4)	726 ± 256 (4)
Alkaline phosphatase[b]	34.5 ± 4.6 (4)	1898 ± 808 (3)
Glutamic oxalacetic transaminase[c]	0.239 ± 0.032 (4)	19.7, 16.0
Lactate dehydrogenase[c]	0.240 ± 0.077 (4)	190 ± 74 (3)
Acetate esterase[c]	18.8 ± 5.7 (4)	17.3 ± 5.4 (4)

[a] Rabbit lungs were lavaged once and cells removed as described under Materials and Methods. Plasma was prepared by sedimentation of cells from heparinized blood. [b] nmol of substrate hydrolyzed per h per mg of protein. [c] nmol per min per mg of protein. [d] Mean ± SD (*N*), where *N* = number of rabbits.

for by their release from ruptured capillaries, at least during the lavage procedure. Only nonspecific esterase has similar specific activities in plasma and lavage effluents.

Some enzymic components of the extracellular lining could arise from plasma via a selective transudation process; however, such a mechanism probably does not account for the presence of β-*N*-acetylglucosaminidase, α-mannosidase, or alkaline phosphatase since the lavage effluent enzymes are electrophoretically distinct from their serumal counterparts (Figure 3).

Both A and B forms of β-*N*-acetylglucosaminidase are present in lavage effluents, but only an A form is present in serum. Also, β-*N*-acetylglucosaminidase of serum appears to have a slightly greater electrophoretic mobility than β-*N*-acetylglucosaminidase A of the lavage effluents; when serum and lavage effluents are mixed prior to electrophoresis, the serumal enzyme can still be located on the gel rods. Although the relative quantities of A and B forms differ, extracellular β-*N*-acetylglucosaminidase is not electrophoretically distinguishable from the corresponding multiple forms present in alveolar macrophages or remaining in lung tissue following the lavage procedure.

α-Mannosidase of cell-free lavage effluents is electrophoretically similar to that found in lung lysosomes and alveolar macrophages. However, serum contains two forms of α-mannosidase, both of which are electrophoretically distinct from the lavage effluent enzyme.

Acid phosphatase of cell-free lavage effluents consists of three electrophoretically distinct multiple forms. The major form appears to be present in serum and alveolar macrophages and may also be present in pulmonary lysosomes, although the lysosomal component is obscured. However, alveolar macrophages, pulmonary lysosomes, and serum contain acid phosphatase multiple forms which are not present in cell-free lavage effluents.

Alkaline phosphatase of cell-free lavage effluents is electrophoretically distinct from that found in serum. Pulmonary lysosomal preparations also appear to contain alkaline phosphatase in which only a minor component has an electrophoretic mobility similar to the extracellular enzyme.

Hydrolases of Lamellar Bodies. Hydrolases have been detected histochemically within lamellar bodies of alveolar type 2 cells (see, for example, Goldfischer et al., 1968) and in preparations of isolated lamellar bodies (DiAugustine, 1974). Consideration of lamellar bodies following their secretion by

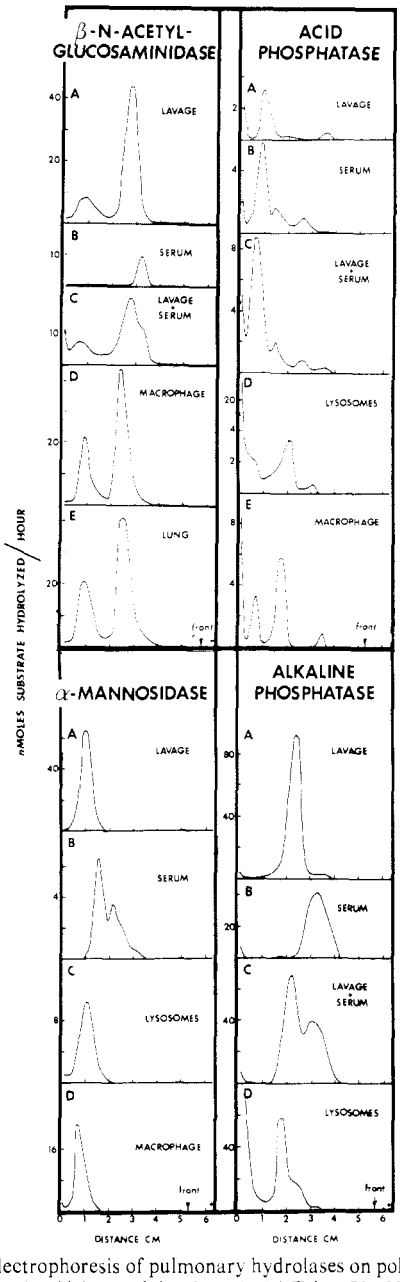

FIGURE 3: Electrophoresis of pulmonary hydrolases on polyacrylamide gel rods (4% acrylamide) containing 0.1% (w/v) Triton X-100. All samples were applied to the gels in the presence of 0.1% Triton X-100. The distance moved by the electrophoretic front was the same for each gel rod within a particular hydrolase group. Following electrophoresis the gel rods were cut into 2-mm sections and assayed for enzyme activity. Cell-free lavage effluents were prepared as described under Materials and Methods. Blood serum was obtained using standard procedures from the same rabbits used for the preparation of lavage effluents. Alveolar macrophages isolated from lavage effluents were homogenized in the presence of 0.1% Triton X-100: protein from the 178 000g_{av} (1 h) supernatant was used in the electrophoresis. Lysosome-rich fractions were prepared from lavaged lungs (see Materials and Methods). β-*N*-Acetylglucosaminidase: (A) cell-free lavage effluent (93 μg of protein); (B) serum (1650 μg of protein); (C) serum (1650 μg of protein) + cell-free lavage effluent (93 μg of protein); (D) alveolar macrophage (22 μg of protein); (E) following 7 lavages, lungs were homogenized in the presence of 0.1% Triton X-100. Protein (123 μg) from the 178 000g_{av} (1 h) supernatant was applied to the gel rod. Acid Phosphatase: (A) cell-free lavage effluent (73 μg of protein); (B) serum (3075 μg of protein); (C) serum (3075 μg of protein) + cell-free lavage effluent (73 μg of protein); (D) lysosome-rich fraction (113 μg of protein); (E) alveolar macrophage (87 μg of protein). α-Mannosidase: (A) cell-free lavage effluent (104 μg of protein); (B) serum (3075 μg of protein); (C) lysosome-rich fraction (113 μg of protein); (D) alveolar macrophage (87 μg of protein). Alkaline Phosphatase: (A) cell-free lavage effluent (104 μg of protein); (B) serum (3075 μg of protein); (C) serum (3075 μg of protein) + cell-free lavage effluent (104 μg of protein); (D) lysosome-rich fraction (160 μg of protein).

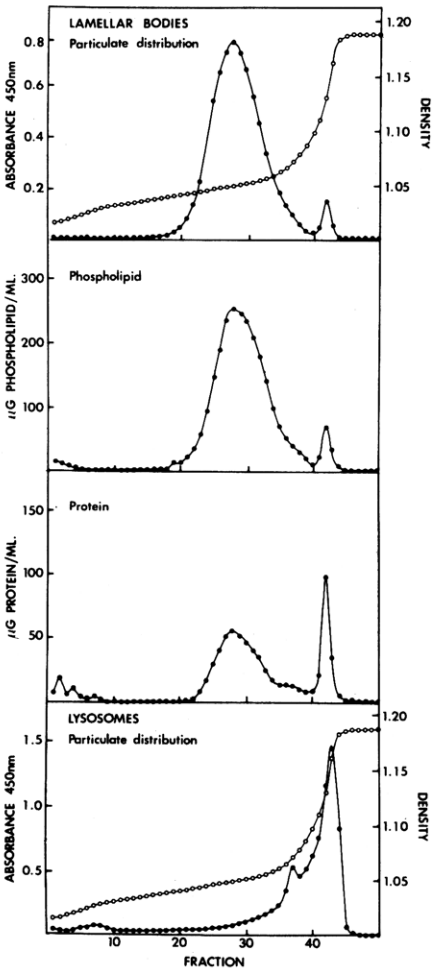

FIGURE 4: Isopycnic centrifugation on continuous sucrose density gradients of lamellar bodies and lysosomes prepared from homogenates of rabbit lung. Distributions of particulate components on the gradients were determined by the absorbance of each fraction at 450 nm. The particulate, phospholipid, and protein distributions were obtained from the same preparation of lamellar bodies.

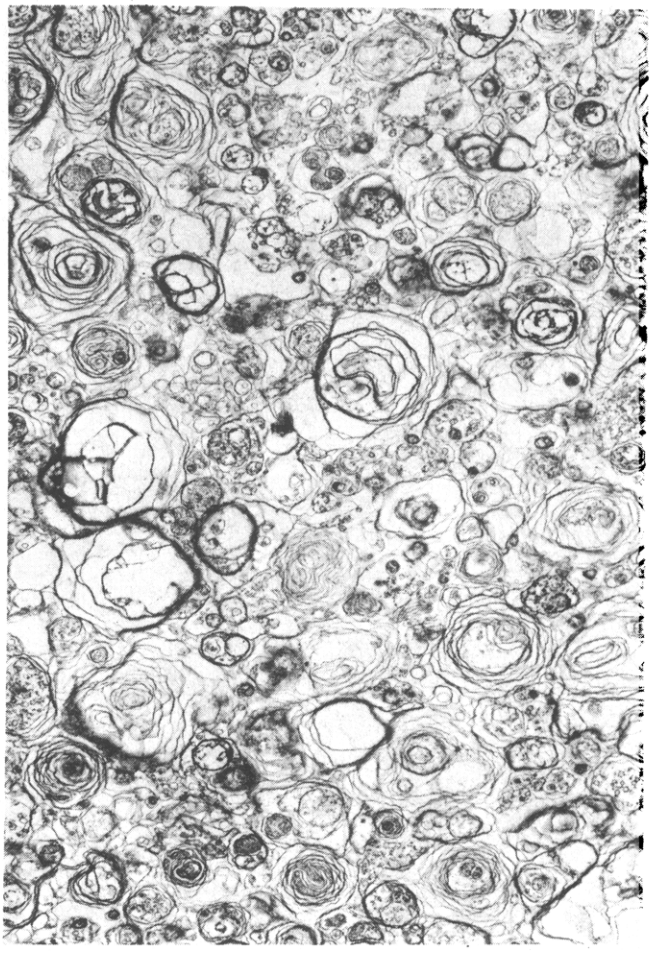

FIGURE 5: Morphology of particulate materials with mid-peak density of 1.048 obtained from continuous sucrose density gradients following the isopycnic centrifugation of lamellar bodies prepared from rabbit lungs (×13 400).

the type 2 cell as a source of the extracellular hydrolases necessitated preparation of the bodies free of contamination with lysosomes. Lamellar bodies free of lysosomes were obtained by subjecting lamellar bodies prepared by the procedure of DiAugustine (1974) to isopycnic centrifugation on sucrose density gradients (Figure 4). The particulate distribution is shown by the absorbance of each fraction at 450 nm: two major particulate components are present with mid-peak densities of 1.048 and 1.133. Phospholipid to protein ratios of the mid-peak fractions are 4.2 and 0.64 for the lower and higher density components, respectively. On identical gradients lysosome-rich fractions prepared from lung homogenates band with the major particulate component, having a density of 1.163. The lysosomal preparation is completely free of lamellar bodies and the lamellar bodies appear to be free of lysosomes.

Electron micrographs of the two major particulate components present in lamellar body preparations and isolated from the sucrose density gradients illustrated in Figure 4 are shown in Figures 5 and 6. In spite of considerable disruption, the material with mid-peak density of 1.048 is clearly lamellar body-derived. Lamellar bodies from the rabbit appear to be sensitive to osmotic changes and mechanical shock; consequently, they are difficult to prepare with well-preserved morphology. The inclusion of Hanks' medium in the sucrose gradients helped protect against the destruction of lamellar bodies during isopycnic equilibration. Lamellar bodies shown in Figure 5 have retained their multilamellated structure to varying degrees. Many of the smaller vesicular structures containing amorphous material are also seen at the center of lamellated whorls of membranes and may represent the core unit often seen in lamellar bodies within the type 2 cell.

The material with mid-peak density of 1.133 consists of extensive sheets of membranes and membranous vesicles; a few lamellar bodies are also present. Much of the material appears to exist in the form of double membranes separated by amorphous matter. The membranes may originate in plasma membranes since both phosphodiesterase IV and alkaline phosphatase were present (G. E. R. Hook, unpublished observations). Regardless of the origins of the higher density material, it is clearly morphologically distinct from lamellar bodies.

The distributions of α-mannosidase and β-*N*-acetylglucosaminidase on continuous sucrose density gradients following isopycnic density gradient centrifugation of lamellar bodies and lysosomes are shown in Figure 7. Both hydrolases are associated with lamellar bodies. Lysosomal contamination appears to be very small if at all present. Hydrolase activity associated with the minor high density component is probably due to its observed contamination with lamellar bodies. Hydrolase activity at the low density end of the gradient is due to the presence of free enzyme.

All of the hydrolases that appear to exist extracellularly are detectable in lamellar bodies. The relative specific activities

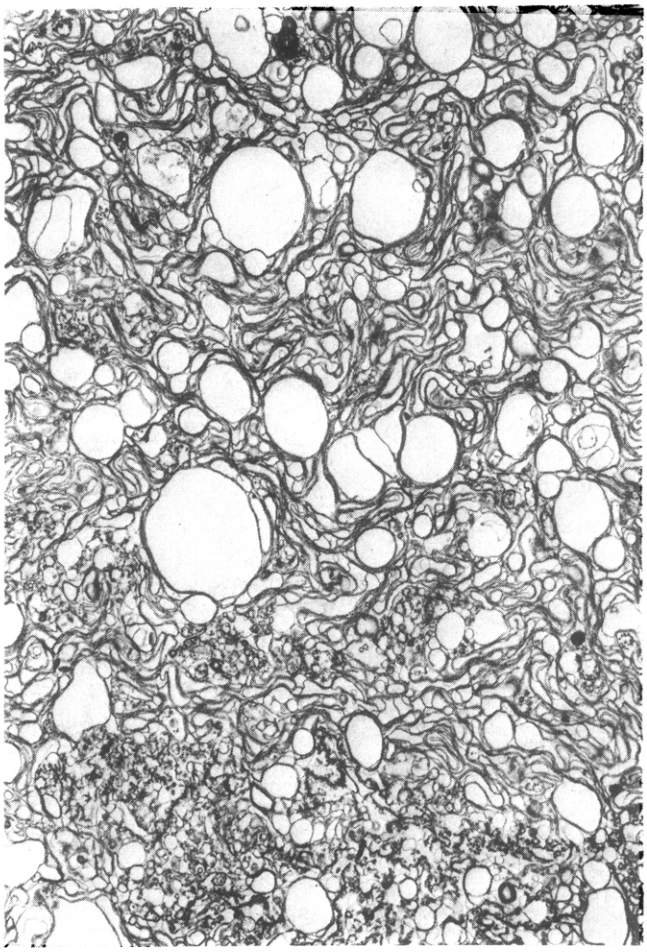

FIGURE 6: Morphology of particulate materials with mid-peak density of 1.133 obtained from continuous sucrose density gradients following the isopycnic centrifugation of lamellar bodies prepared from rabbit lungs (×10 950).

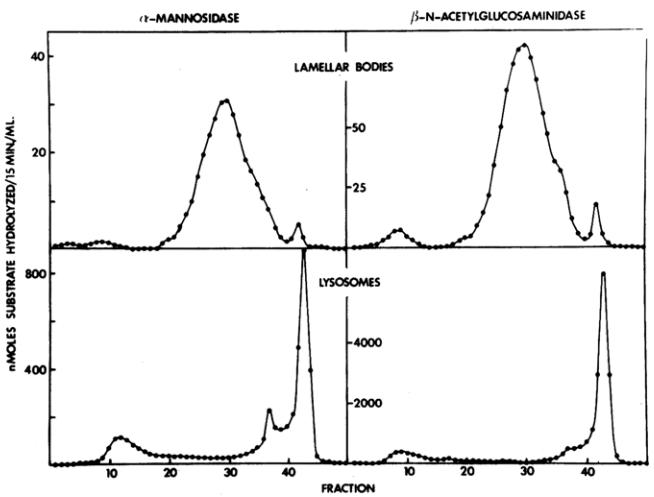

FIGURE 7: The distribution of α-mannosidase and β-*N*-acetylglucosaminidase on continuous sucrose density gradients following isopycnic centrifugation of lamellar bodies and lysosomes prepared from homogenates of rabbit lungs. The gradients are identical with those in Figure 4.

of several hydrolases present within the lamellar bodies isolated following isopycnic centrifugation on continuous sucrose density gradients are shown in Figure 8. As with the cell-free lavage effluents, the most active hydrolases associated with lamellar bodies are in the order β-*N*-acetylglucosaminidase

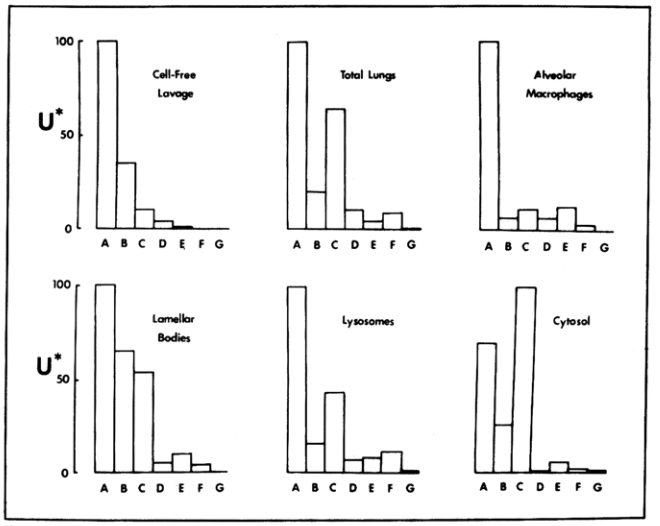

FIGURE 8: Relative specific activities of hydrolases in fractions of the lungs. Lungs were lavaged six times; the lavage effluents were pooled and then centrifuged at $580g_{av}$ for 10 min. The supernatant was labeled "cell-free lavage" and the sediment "alveolar macrophages." Lamellar bodies were isolated free of lysosomes following isopycnic centrifugation of continuous sucrose gradients. Lysosome and cytosol fractions were prepared according to the procedures described under Materials and Methods. (*) Relative specific activities, i.e., the specific activity of the most active hydrolase in each lung fraction was assigned an arbitrary value of 100 and the specific activities of the other hydrolases adjusted accordingly. Particular hydrolases from each fraction of the lung were measured under similar conditions of incubation. (A) β-*N*-Acetylglucosaminidase; (B) α-mannosidase; (C) acid phosphatase; (D) β-galactosidase; (E) fatty acid esterase; (F) arylsulfatase; (G) β-glucuronidase.

> α-mannosidase > acid phosphatase, a particular sequence which is not found in any other hydrolase-bearing fraction which we have been able to isolate from the lungs. The most common sequence lies with acid phosphatase > α-mannosidase and is found in lysosomes, cytosol, alveolar macrophages, and lung homogenates. However, since different inactivation processes could produce changes in the relative specific activities of hydrolases within these pulmonary fractions, the similarity between cell-free lavage and lamellar bodies must be considered with caution.

Discussion

The acid hydrolases investigated in this research are generally considered to be of lysosomal origin at least insofar as the lung (DeLumen et al., 1972) and some nonpulmonary tissues are concerned (Barrett, 1973; Tappel, 1973). Alkaline phosphatase is not a normal lysosomal component, although it has been detected in lysosome-like granules in a few tissues (Horn et al., 1964; Hugon and Borgers, 1966; Novikoff et al., 1965). This report has established the presence of an extracellular pool of hydrolytic enzymes within the airways of the lungs. In addition, this pool of hydrolases appears to be located in the acellular lining of the distal airways, as indicated by the relationship which these hydrolases bear to the phospholipid component of the lining.

Within the acellular lining the hydrolases may exist in two distinct compartments, both of which contain phospholipids. However, over 90% of the hydrolase activity present in lavage effluents is derived from the major compartment. The major and minor compartments, distinguishable by their differing abilities to release hydrolases into the lavage medium, could indicate structural organization of the acellular lining or attempts by the lung to replace lining materials removed by the lavage process.

The cellular origins of the extracellular hydrolases are uncertain. Neither disruption of cells nor leakage of serum can account for the presence of these enzymes in lavage effluents. In general, the multiple forms of hydrolases present in serum appear to be electrophoretically distinct from those present in the acellular lining and, although hydrolases are present in pulmonary supernatant fraction (these hydrolases may leak from lysosomes during preparation of the fraction) (Owens et al., 1975), the extracellular hydrolases and lactate dehydrogenase present in lavage effluents do not arise from the same source. Alveolar macrophages, which have been shown to secrete lysosomal enzymes during phagocytosis (Ackerman and Beebe, 1974, 1975), must be considered a possible source of extracellular hydrolases in the pulmonary airways. However, in the absence of selective hydrolase inactivation processes, mechanisms selective for the secretion of different lysosomal enzymes would have to be involved since the relative abundance of hydrolytic enzymes in alveolar macrophages is different from that in the extracellular pool. Although some extracellular hydrolases may arise from alveolar macrophages, soluble alkaline phosphatase does not since it is absent from this cell (Cohn and Wiener, 1963a) and that which is present in the cellular component of lavage effluents is all membrane-associated (G. E. R. Hook, unpublished observations).

An alternative or at least contributory source of extracellular hydrolases appears to be the type 2 cell. Association of hydrolytic enzymes with the lamellar bodies of the type 2 cell has been noted by histochemists for many years. Acid phosphatase (Balis and Conen, 1964; Corrin et al., 1969; Goldfischer et al., 1968; Hatasa and Nakamura, 1965; Kuhn, 1968; Meban, 1972), arylsulfatase (Corrin and Clark, 1968; Goldfischer et al., 1968; Meban, 1973), phosphatidic acid phosphohydrolase (Meban, 1972), and nonspecific esterase (Hitchcock-O'Hare et al., 1976; Klika and Petrik, 1965; Vatter et al., 1968), enzymes which have all been found in lysosomes, have been detected in lamellar bodies of the type 2 cell using histochemical techniques. Alkaline phosphatase has also been detected histochemically in lamellar bodies (Buckingham et al., 1964; Klika and Petrik, 1965; Kuhn, 1968). Our studies have shown a number of hydrolases to be associated with lysosome-free, highly purified preparations of lamellar bodies. The presence of hydrolases in lamellar bodies, especially those hydrolases most active in the extracellular lining of the pulmonary airways, and the general concurrence of opinion concerning the secretion of lamellar bodies by the type 2 cells, identifies this alveolar epithelial cell as a presumptive, although not necessarily only, source of the extracellular hydrolases.

Lysosomal enzymes are not usually secreted from mammalian cells, although a number of exceptions have been reported: cells above the stratum granulosum of the skin epidermis (Wolff and Schreiner, 1970), cartilaginous limb-bone rudiments of the embryonic chick grown in organ culture (Dingle, 1973), cells of the adrenal medulla during exocytosis of catecholamines (Schneider, 1970), osteoclasts during parathyroid hormone-induced bone resorption (Vaes, 1973), platelets (Holmsen and Day, 1970), fibroblasts (Werb and Dingle, 1976), polymorphonuclear leukocytes (Crowder et al., 1969), and macrophages (Cohn and Wiener, 1963b) all secrete or can be induced to secrete lysosomal enzymes. The secretion of lysosomal enzymes, while often observable under in vitro conditions such as in cell or organ culture, is difficult to observe in vivo. This report describes for the first time the extent of an extracellular pool of hydrolytic enzymes in any tissue.

In view of the potential of extracellular lysosomal enzymes to damage tissue constituents, the secretion of these enzymes into the airways of the lungs would appear to be an undesirable process. However, it is apparent from our limited studies that the extracellular hydrolases are of a selected variety. While some hydrolases such as α-mannosidase and β-*N*-acetylglucosaminidase are highly active in the acellular lining, others such as arylsulfatase, β-glucuronidase, and fatty acid esterase are barely detectable and neuraminidase and phospholipase appear to be absent (G. E. R. Hook, unpublished observations). Consequently, lysosomal enzymes in the airways of the lungs may serve specific functions unrelated to their digestive functions within the cell.

Acknowledgments

We thank Robert E. Easterling for technical assistance, Fred A. Talley for assistance with the electron microscopy, Dr. Joseph K. Haseman for the statistical analyses, Dr. James R. Fouts for encouragement and support, and Dr. R. P. DiAugustine for assistance and many hours of stimulating discussion.

References

Ackerman, N. R., and Beebe, J. R. (1974), *Nature (London) 247,* 475.

Ackerman, N. R., and Beebe, J. R. (1975), *J. Pharmacol. Exp. Ther. 193,* 603.

Balis, J. U., and Conen, P. E. (1964), *Lab. Invest. 13,* 1215.

Barrett, A. J. (1973), in Lysosomes in Biology and Pathology, Vol. 2, Dingle, J. T., and Fell, H. B., Ed., New York, N.Y., Elsevier, p 245.

Bergmeyer, H.-U. (1965), in Methods of Enzymatic Analysis, Bergmeyer, H.-U., Ed., New York, N.Y., Academic Press, p 976.

Bhattacharyya, S. N., Passero, M. A., DiAugustine, R. P., and Lynn, W. S. (1975), *J. Clin. Invest. 55,* 914.

Bignon, J., Chahinian, P., Feldmann, G., and Sapin, C. (1975), *J. Cell Biol. 64,* 503.

Bignon, J., Juarand, M. C., Pinchon, M. C., Sapin, C., and Warnet, J. M. (1976), *Am. Rev. Respir. Dis. 113,* 109.

Buckingham, S., McNary, W. F., Jr., and Sommers, S. C. (1964), *Science 145,* 1192.

Cohn, Z. A., and Wiener, E. (1963a), *J. Exp. Med. 118,* 991.

Cohn, Z. A., and Wiener, E. (1963b), *J. Exp. Med. 118,* 1009.

Conchie, J., and Hay, A. J. (1959), *Biochem. J. 73,* 327.

Corrin, B., and Clark, A. E. (1968), *Histochemie 15,* 95.

Corrin, B., Clark, A. E., and Spencer, A. (1969), *J. Anat. 104,* 65.

Crowder, J. G., Martin, R. R., and White, A. (1969), *J. Lab. Clin. Med. 74,* 436.

DeLumen, B. O., Taylor, S., Urribarri, N., and Tappel, A. L. (1972), *Biochim. Biophys. Acta 268,* 597.

DiAugustine, R. P. (1974), *J. Biol. Chem. 249,* 584.

Dingle, J. T. (1973), in Lysosomes in Biology and Pathology, Vol. 2, Dingle, J. T., and Fell, H. B., Ed., New York, N.Y., Elsevier, p 421.

Dodgson, K. S., Spencer, R., and Thomas, J. (1955), *Biochem. J. 59,* 29.

Folch, J., Lees, M., and Stanley, G. H. S. (1957), *J. Biol. Chem. 226,* 497.

Gianetto, R., and DeDuve, C. (1955), *Biochem. J. 59,* 433.

Goldfischer, S., Kikkawa, Y., and Hoffman, L. (1968), *J. Histochem. Cytochem. 16,* 102.

Green, S. F., Cantor, F., Inglis, N. R., and Fishman, W. H. (1972), *Am. J. Clin. Pathol. 57,* 52.

Hand, W. L., and Cantey, J. R. (1974), *J. Clin. Invest. 53,* 354.

Hanks, J. H., and Wallace, R. E. (1949), *Proc. Soc. Exp. Biol. Med. 71,* 196.
Hatasa, K., and Nakamura, T. (1965), *Z. Zellforsch. Mikrosk. Anat. 68,* 266.
Hitchcock-O'Hare, K., Maymaris, E., Bonaccorso, J., and VanBuren, S. G. (1976), *J. Histochem. Cytochem. 24,* 487.
Holmsen, H., and Day, H. J. (1970), *J. Lab. Clin. Med. 75,* 840.
Hook, G. E. R., Bend, J. R., Hoel, D., Fouts, J. R., and Gram, T. E. (1972), *J. Pharmacol. Exp. Ther. 182,* 474.
Horn, R. G., Spicer, S. S., and Wetzel, B. K. (1964), *Am. J. Pathol. 45,* 327.
Howard, R. B., Lee, J. C., and Pesch, L. A. (1973), *J. Cell Biol. 57,* 642.
Hugon, J., and Borgers, M. (1966), *J. Histochem. Cytochem. 14,* 629.
Klika, E., and Petrik, P. (1965), *Acta Histochem. 20,* 331.
Kuhn, C. (1968), *Am. J. Pathol. 53,* 809.
Levvy, G. A., and McAllen, A. (1963), *Biochem. J. 87,* 361.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Luft, J. H. (1961), *J. Biophys. Biochem. Cytol. 9,* 409.
Macklem, P. T., Proctor, D. F., and Hogg, J. C. (1970), *Respir. Physiol. 8,* 191.
Mahadevan, S., and Tappel, A. L. (1968), *Arch. Biochem. Biophys. 126,* 945.
Meban, C. (1972), *J. Cell Biol. 53,* 249.
Meban, C. (1972), *J. Anat. 293,* 302.
Meban, C. (1973), *Histochem. J. 5,* 557.
Novikoff, A. B., Iaciofano, P., and Villaverde, H. (1965), *J. Histochem. Cytochem. 13,* 29.
Owens, J. W., Gammon, K. L., and Stahl, P. D. (1975), *Arch. Biochem. Biophys. 166,* 258.
Pattle, R. E. (1958), *Proc. R. Soc. London 148,* 217.
Reasor, M. J., Nadeau, D., and Hook, G. E. R. (1978), *J. Appl. Physiol.* (in press).
Reynolds, E. S. (1963), *J. Cell Biol. 17,* 208.
Reynolds, H. Y., and Newball, H. H. (1974), *J. Lab. Clin. Med. 84,* 559.
Schneider, F. H. (1970), *Biochem. Pharmacol. 19,* 833.
Schwartz, M. K., and Bodansky, O. (1966), *Methods Enzymol. 9,* 294.
Sellinger, O. Z., Beaufay, H., Jaques, P., Doyan, A., and De-Duve, C. (1960), *Biochem. J. 74,* 450.
Shin, Y. S. (1962), *Anal. Chem. 34,* 1164.
Tappel, A. L. (1973), in Lysosomes in Biology and Pathology, Vol. 2, Dingle, J. T., and Fell, H. B., Ed., New York, N.Y., Elsevier, p 207.
Turnbull, J. M., and Neil, M. W. (1969), *Biochem. J. 111,* 503.
Vaes, G. (1973), in Lysosomes in Biology and Pathology, Vol. 1, Dingle, J. T., and Fell, H. B., Ed., New York, N.Y., Elsevier, p 217.
Vatter, A. E., Reiss, O. K., Newman, J. K., Lindquist, K., and Groeneboer, E. (1968). *J. Cell Biol. 38,* 80.
Watson, M. L. (1958), *J. Biophys. Biochem. Cytol. 4,* 475.
Werb, Z., and Dingle, J. T. (1976), in Lysosomes in Biology and Pathology, Vol. 5, Dingle, J. T., and Dean, R. T., Ed., New York, N.Y., Elsevier, p 127.
Williams, M. C. (1977), *J. Cell Biol. 72,* 260.
Wolff, K., and Schreiner, E. (1970), *Arch. Dermatol. 101,* 276.

Transient-State Kinetics of L-Glutamate Dehydrogenase: Mechanism of α-Ketoglutarate Inhibition in the Burst Phase†

Alan H. Colen

ABSTRACT: Stopped-flow studies of the initial burst of NADPH production accompanying the oxidative deamination of L-glutamate by L-glutamate dehydrogenase and $NADP^+$ were performed in the presence of α-ketoglutarate, a product of the reaction. Both binary enzyme-α-ketoglutarate and ternary enzyme-$NADP^+$-α-ketoglutarate complexes are inhibitory in the burst phase of the enzyme-catalyzed reaction. Order-of-addition experiments show the binary complex to form rapidly, in the 3 ms dead time of the stopped-flow instrument. There is a distinct lag, however, in the achievement of the full ternary complex inhibitory effect unless the enzyme is preincubated with both $NADP^+$ and α-ketoglutarate prior to initiation of the catalytic reaction with L-glutamate. The formation of an inhibitory enzyme-$NADP^+$-α-ketoglutarate complex appears to be sufficiently slow to give a delayed kinetic response when α-ketoglutarate is added to the reaction system.

Studies of the steady-state kinetics of the oxidative deamination of L-glutamate by L-glutamate dehydrogenase and NAD^+ have revealed that glutarate and the product, α-ketoglutarate, are potent competitive inhibitors of L-glutamate oxidation (Caughey et al., 1957). In stopped-flow studies, it has been possible to isolate the very initial steps of L-glutamate oxidation by L-glutamate dehydrogenase (Iwatsubo and Pantaloni, 1967; Fisher et al., 1970; di Franco and Iwatsubo, 1971, 1972; Colen et al., 1972; Fisher, 1973; di Franco, 1974;

† From the Laboratory of Molecular Biochemistry, Veterans Administration Hospital, Kansas City, Missouri 64128, and the University of Kansas Medical School, Kansas City, Kansas. *Received February 5, 1976; revised manuscript received July 14, 1977.* This investigation was supported in part by grants to Dr. Harvey F. Fisher from the National Science Foundation (BMS75-17107) and the General Medicine Institute of the National Institutes of Health (GM-15188). A preliminary report of this work was presented at the 1975 FASEB Annual Meeting in Atlantic City, New Jersey (Colen, 1975).

0006-2960/78/0417-0528$01.00/0