

ENZYMES OF THE LUNG

I. Detection of Esterase with a New Cytochemical Method

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ABSTRACT

The esterases of rabbit lung have been investigated from two viewpoints, the cytochemical and the biochemical. To accomplish this objective, we designed and synthesized a series of ester substrates which provide both a cytochemical indicator of the location of the enzyme and a means of following the enzymatic activity in tissue homogenates and subfractions. The substrates are *p*-nitrophenylthiol esters which yield, upon hydrolysis, carboxylic acid and *p*-nitrothiophenol. The latter can react with aurous ions to give an electron-opaque deposit; in addition, the strong absorption of *p*-nitrothiophenol at 410 m μ permits continuous kinetic measurements. Thus, it is possible to correlate the intracellular site of action and the biochemical behavior of the esterases. The new substrates are the thiol analogues of the *p*-nitrophenyl esters frequently employed as esterase substrates. The rates of hydrolysis of the two series of esters are compared in vitro. During tissue fractionation, most of the esterase activity sediments with a particulate fraction. The effects of a number of common esterase inhibitors, such as diisopropyl phosphorofluoridate and eserine sulfate, are examined, and the effects of enzyme concentration and heat inactivation are shown with the use of the partially purified preparations. The cytochemical work shows that the esterase activity is most prominent in the lamellar bodies of the giant alveolar (type II, septal, or granular pneumatocyte) cells of the lung and to a lesser extent in squamous (type I, or membranous pneumatocyte) epithelial and endothelial cells. In both the cytochemical and biochemical studies, the enzymes are inhibited by diisopropyl phosphorofluoridate and phenyl methylsulfonyl fluoride but are insensitive to eserine sulfate.

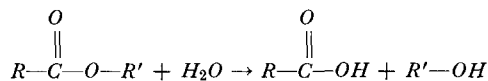
INTRODUCTION

The enzymatic profiles of lung are quantitatively different from those of liver or kidney (1). Other investigators have observed (2-6), and our studies confirm, that the lung is rich in hydrolytic activity; therefore we began our researches in this area.

Carboxylic ester hydrolase (3.1.1)¹ is defined

¹ We shall refer to this hydrolytic activity as esterase throughout this report, since we do not know whether

as an enzyme that will catalyze the following reaction:



we are dealing with a carboxyl ester hydrolase (3.1.1), a thiol ester hydrolase (3.1.2), or a peptide hydrolase (3.4). See Discussion.

Past studies of esterases have fallen into two main types, the histochemical work and the biochemical studies of tissue fractions.

Gomori (7) has reviewed the earlier histochemical work. In general, the approach in all these studies has been to employ a synthetic substrate which, upon hydrolysis, will yield a product that can be directly observed at the site of action with the microscope. Burstone has reviewed the use of many of these substrates (8).

The earliest practical histochemical procedure was that of Gomori (9). His work led to the use of α - and β -naphthyl acetates, indoxyl esters and similar compounds as substrates. Upon hydrolysis, the freed, substituted naphthol or indoxyl is converted into a dye (10, 11). Koelle and Friedenwald (12) introduced a method which utilized thiocholine. The carboxylic esters of thiocholine sulfate are hydrolyzed, and the liberated thiocholine sulfate is precipitated in the presence of copper salts. This reaction forms the basis of the electron microscopic method, whereby the "capturing agent," usually copper or lead, combines with the product to yield an opaque precipitate (13).

Wilson (14) introduced the use of thiolacetic acid as a substrate for esterase and indicated that an esterase enzyme had split this molecule into acetic acid and hydrogen sulfide which could be precipitated in the presence of lead or copper. Barrnett adapted this technique for electron microscopy (15). Koelle and Foroglou-Kerameos (16) used aurous ions as the heavy metal and found that this procedure yields a finer reaction product (17).

The biochemical studies, on the other hand, have concentrated on the esterases in body fluids and in subcellular fractions of tissues. These *in vitro* studies have employed esters of *p*-nitrophenol (PNP) and similar compounds as substrates because the hydrolytic activity can be followed spectrophotometrically. Rates of hydrolysis, effects of enzyme and substrate concentrations, and pH effects were thus measurable *in vitro*. Huggins and Lapidus (18) measured the activity of human serum enzyme, and other workers have used similar substrates on serum (19), subcellular fractions of brain (20, 21), and human gastric juice (22, 23).

For sorting out the different types of esterases and assigning them places and functions within the cell, a single substrate which can act both as a histochemical and as a biochemical detecting

agent would be ideal. We have attempted to achieve this objective in the present study in order to correlate structure and function.

MATERIALS AND METHODS

Chemical Syntheses

p-NITROTHIOPHENOL (PNT): *p*-Nitrothiophenol was prepared according to the method of Brown and Rao (24).

6 g of recrystallized bis-(*p*-nitrothiophenyl) disulfide (mp, 181–182° C) (Aldrich Chemical Co., Milwaukee, Wis.) were reduced by 1 g of sodium borohydride dissolved in 25 ml of redistilled diglyme (diethylene glycol dimethyl ether; The Ansul Co., Marinette, Wis.) and 0.9 g of freshly sublimed AlCl_3 suspended in 45 ml of redistilled diglyme. After 1 hr, 50 ml of 2 M HCl were added slowly to destroy the excess hydride. The yellow PNT separated after cooling on an ice bath and was washed with water. The yield was 88% with mp 78.5°–79.5° C (Table I). The product showed spontaneous slow oxidation in air to the disulfide.

p-NITROPHENYLTHIOL ACETATE (PNTA): 10 ml of pyridine were treated with a bubbling stream of N_2 for 2 or 3 min. 500 mg of PNT were dissolved in the pyridine, and 3 ml of acetyl chloride (Mallinckrodt Chemical Works, St. Louis, Mo.) were added dropwise, with stirring, to the flask which was kept in an ice bath. The reaction mixture was allowed to stand for 5 min, and then 50 ml of cold distilled water were added to it; this dissolved the pyridine hydrochloride and precipitated the PNTA. The mixture was allowed to stand in ice for 1 hr, and the precipitate was recovered by filtration, washed with cold water, and dried; a 44% yield of pale yellow crystals that melted at 78°–79° C was obtained.

This product is recrystallized from water to give very fine needles which melt at 81.5°–82° C.

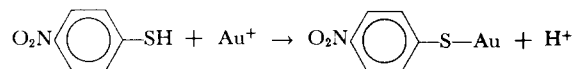
The ester was examined by means of thin-layer chromatography (see below) and gave a single spot with an R_f value distinctly different from the R_f values of the disulfide and the starting material (Table I).

p-NITROPHENYLTHIOL BUTYRATE (PNTB): 4 ml of dry pyridine were added to 100 ml of anhydrous benzene. 5 g of PNT were added, and then 10 ml of freshly distilled butyryl chloride were added slowly. The mixture was refluxed for 2 hr and was allowed to stand overnight. The pyridine hydrochloride was removed by filtration, and the clear solution was washed successively twice with water, 2% NaHCO_3 , and water again. The solution was then dried over Na_2SO_4 , and the benzene was evaporated from the product under vacuum. The remaining mixture of liquid and solid was extracted twice

with ether; the extracts were pooled, the ether was evaporated, and the brown oil that remained was redistilled in a microdistillation apparatus. The ester distilled at 115° C, 2–3 mm.

Other homologous esters, such as the propionate and octanoate, were prepared by the same method as that used for PNTB.

AUROS *p*-NITROTHIOLPHENOLATE: The compound was formed according to the following equation:



250 mg of $\text{AuNa}_3(\text{S}_2\text{O}_3)_2 \cdot 2\text{H}_2\text{O}$ (Sanocrysin; Ferrosan, Copenhagen, Denmark) were dissolved in 6.0 ml of 0.175 M sodium maleate buffer pH 7.4 and added to 52.2 mg of PNT dissolved in 40 ml of water. After it had stood for 60 min, a yellow precipitate formed. The precipitate was washed by centrifugation twice with cold H_2O , twice with dry methanol, once with ethyl acetate, and once with ethyl ether. It was dried 1 hr *in vacuo* over P_2O_5 . 60.6 mg, 173 μmoles , were recovered for a yield of 51%. Analysis was as follows: calculated, Au, 56.09%; C, 20.52%; H, 1.15%; S, 9.13%; found; Au, 55.52%; C, 20.82%; H, 1.28%; S, 9.24%. The mercaptide is insoluble in alcohol, ethyl acetate, and ethyl ether, but is freely soluble in pyridine, like the mercuric *o*-thiophenolate (25).

CHARACTERIZATION OF COMPOUNDS: All compounds described above were also examined by thin layer chromatography on silica gel HF₂₅₄ (Brinkman Laboratories, Inc., Great Neck, N. Y.) plates; a solvent system of hexane:benzene:acetic acid, 50:50:0.5, was used, and the spots were read under a short wave ultraviolet lamp. The absorption spectra were determined in the ultraviolet and visible regions on a Cary 14 recording spectrophotometer in several solvents and in the infrared region in potassium bromide micropellets 1 × 5 mm in the Beckman IR 7 infrared spectrophotometer.

Preparation of Homogenate and Fractions

Adult rabbits (New Zealand) of either sex and weighing between 1.6 and 2.8 kg were sacrificed by injecting air into the ear vein. The lungs were removed immediately, a portion of a lobe was tied off, and the distal section was removed and used for electron microscopy. The remainder of the lung was used to prepare a homogenate in 0.25 M sucrose, 0.01 M tris-HCl, and 0.001 M potassium EDTA (ethylenediaminetetraacetate). Other details of the

procedure have been described previously (1). After the homogenate had been strained through bolting cloth and centrifuged at 1600 *g* for 5 min for removal of nuclei, whole cells, and debris, the supernatant fraction was centrifuged at 100,000 *g* for 30 min, in the Spinco Model L rotor No. 50 so that a pellet and supernatant fraction would be obtained. The pellet fraction, which under these conditions contains the mitochondria and vesiculated membranes was resuspended in the sucrose-Tris-EDTA solution

and stored at 2°C. All analyses were done within 72 hr; however, the enzymatic activity of both fractions was stable for at least 1 wk.

Inhibitors

Diisopropyl phosphorofluoridate (DFP) (Sigma Chemical Co., St. Louis, Mo.) was prepared as 0.1 M solution by dilution with anhydrous propylene glycol (Distillation Products Industries, Rochester, N. Y.) and stored at 0°C for up to 4 wk from the time of preparation. After 4 wk of storage, the effectiveness of the stock solution in inhibiting the hydrolysis of PNPA (*p*-nitrophenyl acetate) by α -chymotrypsin had decreased less than 10%.

Eserine sulfate (Sigma Chemical Co.) and ambenonium chloride (N,N'-bis-[diethylaminoethyl] oxamide bis-[2-chlorobenzyl chloride])² were prepared as 0.1 M stock solution in 0.175 M sodium maleate and used the same day.

BW 284 (1,5-bis [4-allyl dimethyl ammonium phenyl] pentan 3-one dibromide)³ (26) was prepared as 0.1 M stock solution in the maleate buffer.

Phenyl methylsulfonyl fluoride (PMSF, Sigma Chemical Co.) was prepared as 0.1 M stock solution in the maleate buffer (27).

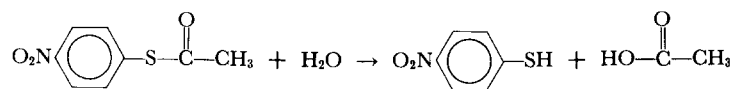
Analytical Methods

Protein contents of the various fractions were determined by a modified Folin procedure (28) with crystalline bovine plasma albumin (Armour Pharmaceutical Co., Chicago, Illinois) as a standard. Nesslerization after complete digestion was employed to standardize this procedure (29).

² We wish to thank the Sterling-Winthrop Research Institute, Rensselaer, N. Y. for a gift of this compound.

³ This compound was kindly donated by Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

RATES OF HYDROLYSIS IN VITRO: The following hydrolytic reaction takes place in the tissue:



Analogous reactions take place with the other esters.

The appropriate ester, 2.5 mg, was dissolved in 1.0 ml of acetonitrile (reagent grade, Fisher Scientific Company, Pittsburgh, Pa.) and then 9.0 ml of water were added. 0.3 ml of this solution was used to initiate the reaction by adding it to 2.7 ml of 0.175 M sodium maleate buffer pH 7.4 containing 0.01–0.1 ml of enzyme in sucrose-Tris medium. Reactions were determined in a final volume of 3.0 ml in a Beckman DU spectrophotometer, coupled through an energy-recording adapter (Beckman No. 5800) to a strip-chart recorder. Reaction rates were evaluated at room temperature, 23°C, as the linear increase in absorbance at 410 m μ between 30 and 120 sec. After initiation of the reaction, the reaction rates remained constant for a minimum of 3 min. In all experiments, less than 40% of the substrate was hydrolyzed in 3 min.

MOLAR ABSORPTIVITY: The molar absorptivity of the crystalline *p*-nitrothiophenol was determined in 0.175 M sodium maleate or in 0.1 M phosphate buffer pH 7.4; at 410 m μ it is 1.25×10^4 cm²/mmole.

INHIBITORS AND HEAT INACTIVATION: The inhibitor to be tested was added to the buffer-enzyme mixture in the cuvette, and the mixture was permitted to stand for 5 min before the reaction was initiated by the addition of the substrate. The rate of heat inactivation of the enzyme was examined as follows: the enzyme-buffer mixture was placed in a water bath at the specified temperatures. At the end of 10 min, the test tubes were transferred to a water bath at room temperature, and the rates were determined after temperature equilibration.

Cytochemical Methods

TISSUE PREPARATION: Slices of lung tissue 1 mm thick were placed in the formaldehyde (4.0%)–sucrose (0.25 M)–sodium maleate (0.028 M) fixative, pH 7.4, at 4°C and deaerated by vacuum. The formaldehyde was freshly prepared from paraformaldehyde (Matheson, Coleman, and Bell, Cincinnati, Ohio). After 15 min fixation, the tissue strips were mounted in 6% agar at approximately 45°C, chilled for 5 min, and cut into 40- μ sections according to the method of Smith and Farquhar (30). These sections were returned to formaldehyde fixative for an additional 30 min at 4°C and were then incubated with substrates. When *s*-collidine buffer was used, irregular reactions were observed. Glutaraldehyde-fixed sec-

tions gave inconsistent reactions unless they were washed thoroughly in several changes of buffer or

were washed overnight. Specimens which were fixed and then kept cold for 24 hr in the formaldehyde fixative or buffer gave the same distribution of cytochemical product as freshly prepared specimens. Also, storing tissues in the cold for 18 hr prior to fixation did not appear to change the reaction pattern.

The following method (16) was utilized for cytochemical location of esterase activity, and the following stock solutions were employed. (a) Thiol ester, 0.025 M, dissolved in dimethylsulfoxide (DMSO). The substrates used in this study were PNTA and PNTB. Thiolacetic acid was also used for comparison. (b) Sodium maleate buffer, 0.175 M, containing 0.25 M NaCl, 0.025 M KCl, 0.025 M CaCl₂, and 0.005 M MgCl₂ and adjusted to pH 7.4 with 1 N NaOH. (c) Sodium aurous thiosulfate, 0.02 M in the above buffer.

After fixation, the tissues were transferred for incubation to a solution containing 1.58 ml of maleate buffer, 0.12 ml of substrate solution, and 0.30 ml of gold thiosulfate solution. The contents were mixed thoroughly and allowed to react at room temperature for 15–60 min. Controls were made by omitting the appropriate reagent and adjusting to a final volume of 2 ml with maleate buffer, and also by using tissues that had been heated at 75°C for 10 min before placing them in the gold-substrate mixture. The effects of several known esterase inhibitors were studied.

The following solutions were used: (a) DFP, 0.1 M in propylene glycol; (b) eserine sulfate, 0.1 M in buffer; (c) PMSF, 0.01 M in isopropanol; (d) ambenonium chloride, 0.01 M in buffer; (e) BW 284, 0.01 M in buffer. For inhibition studies, sufficient reagent was used to give a final inhibitor concentration of 0.001 M in the reaction mixture of 2 ml, except for eserine sulfate which had a final concentration of 0.01 M. Because DFP is not stable in aqueous media, 20 μ l of this inhibitor were added initially and every 15 min during the incubation period. The order of addition of reagents to the tissue was as follows: buffer, inhibitor, sodium aurous thiosulfate, and finally, substrate.

After incubation, the sections were postfixated for 20 min at 4°C in 1% OsO₄ in maleate buffer and then were treated with 2% aqueous uranyl acetate for 45 min at 4°C.

A rinse step between incubation and postossification for removal of soluble gold compounds was unnecessary. Samples were regularly postossified and treated with uranyl acetate to obtain superior preser-

TABLE I
Physical Constants of *p*-Nitrothiophenol and Derivatives

	Melting point		Infrared Absorption				
	Found	Literature*	Thin-layer chromatography†	Aryl-NO ₂ all strong§	$\text{--}\overset{\text{O}}{\parallel}\text{C--S--}^{**}$	$\overset{\text{O}}{\parallel}\text{C--}$ all strong**	Others
<i>p</i> -Nitrothiophenol	78.5-79.5	77	0.76	1340, 1505	none	none	2550¶ (weak)
bis (<i>p</i> -nitrophenyl) disulfide	181.5	168-170, 181	0.58	1345, 1510	none	none	
<i>p</i> -Nitrophenylthiolacetate	81.5-82		0.40	1345, 1510	953	1718	
<i>p</i> -Nitrophenylthiolbutyrate	B.P. 115 (2-3 mm)		0.43	1342, 1515, 1522	970	1717	
Aurous <i>p</i> -nitrothiophenolate				1341, 1518	none	none	

* Beilstein, F. K. 1918. Handbuch der Organischen Chemie. Julius Springer, Berlin. **6**: 339, 340.

† Solvent System: hexane:benzene:acetic acid (50:50:0.5)

§ These are characteristic of aromatic nitro substitution.

** Nyquist and Potts (32) are authority for these assignments.

¶ This is characteristic of --SH (33).

vation and contrast. The use of osmium tetroxide or uranyl acetate did not change the interpretation of the gold deposits.

The specimens were dehydrated in hydroxypropyl methacrylate (Rohm & Haas Co., Philadelphia, Pa.) (31) and embedded in Epon. They were sectioned with a Porter-Blum MT-2 microtome, examined, and photographed with a Philips EM 200 electron microscope. It was not necessary to treat the tissue sections with lead stain.

OBSERVATIONS

Chemical

Table I summarizes some of the physical constants determined. Where possible, these constants have been compared with values from the literature. The synthesized compounds migrated as single spots on the thin-layer plates. After standing, PNT gives an additional spot due to disulfide formation.

The absorption peaks in the infrared region clearly confirm the presence of the postulated functional groups. All compounds contain the characteristic strong absorptions at approximately 1340 and 1520 cm^{-1} of the aryl nitro groups.

The carbonyl stretching frequency, strong evidence for the presence of the ester group, is found at 1718 cm^{-1} in the acetate compound and at 1717 cm^{-1} in the butyrate compound. No similar absorption was found in the spectra of the other nonester compounds examined. The carbon-sulfur stretch, as described by Nyquist and Potts (32), was found at 953 cm^{-1} for the acetate and at 970 cm^{-1} for the butyrate. No carbon-sulfur stretch absorptions are observed for the other compounds shown, since it is the carbonyl carbon that leads to this absorption. As expected, no absorption is found for the disulfide bond, but the weak band cited by Bellamy (33) as characteristic of the sulfhydryl group is observed in the *p*-nitrothiophenol at 2550 cm^{-1} , within the expected region.

Biochemical Observations

In the lung homogenate, the rates of hydrolyses for the thiol esters are of the same order as those for their oxy-analogs, as seen in Table II. Non-enzymatic hydrolysis was less than 10% of the observed rate in all cases. Thin-layer chromatography of the homogenate after 20 min incubation showed additional spots with R_f values similar to those of PNT (0.76) and bis-(*p*-nitrophenyl) disulfide (0.58). This finding indicates that the

TABLE II
Rates of Hydrolysis of Acyl Esters by
Lung Homogenates

Substrate	Sodium maleate buffer	Sodium phosphate buffer
	$\mu\text{mole product}/\text{mg N/min}$	$\mu\text{mole product}/\text{mg N/min}$
<i>p</i> -Nitrophenyl acetate	0.49*	0.59
<i>p</i> -Nitrophenylthiol acetate	0.46	0.57
<i>p</i> -Nitrophenyl butyrate	1.23	1.93
<i>p</i> -Nitrophenylthiol butyrate	2.24	3.32

The assay mixture contained 2.7 ml of 0.175 M sodium maleate or 0.10 M sodium phosphate pH 7.4 and 0.010–0.10 ml of enzyme in the sucrose-Tris medium. The reactions were initiated by the addition of 75 μg of the ester in 0.30 ml water containing 10% acetonitrile. Rates were obtained at 410 μm at room temperature (23°).

The rate of *p*-nitrophenol production was calculated from the molar absorptivity at 410 μm of *p*-nitrophenol or *p*-nitrothiophenol which was found to be $1.25 \times 10^4 \text{ cm}^2 \text{ mmole}^{-1}$ at pH 7.4 in either the maleate or phosphate buffer.

* These values are the average rates obtained from three separate experiments. Rates obtained in each experiment deviated less than 10% from the reported values. The nonenzymatic rates of hydrolysis in either maleate or phosphate buffer varied slightly with the substrate but was in all cases less than 10% of the rates observed in the presence of the enzyme and was subtracted from the reported values.

product of the enzymatic hydrolysis is PNT. As mentioned above, the PNT is quite labile and oxidizes spontaneously in air to form the corresponding disulfide.

ANALYSIS OF FRACTIONS: The pellet and supernatant fractions made from the homogenate showed the distribution of activities given in Table III. The specific activity of the pellet fraction indicates that this fraction has a 3–5-fold purification over the homogenate. The data here suggest that about $\frac{1}{2}$ of the total activity is present in the pellet, and $\frac{1}{2}$ of it is in the supernatant fraction. The recovery of total protein from the fractions of the homogenate is good; the total esterase activity recovered ranges from 78 to 97%.

Fig. 1 *a* and *b* shows that the liberation of PNT is linearly proportional to the pellet protein con-

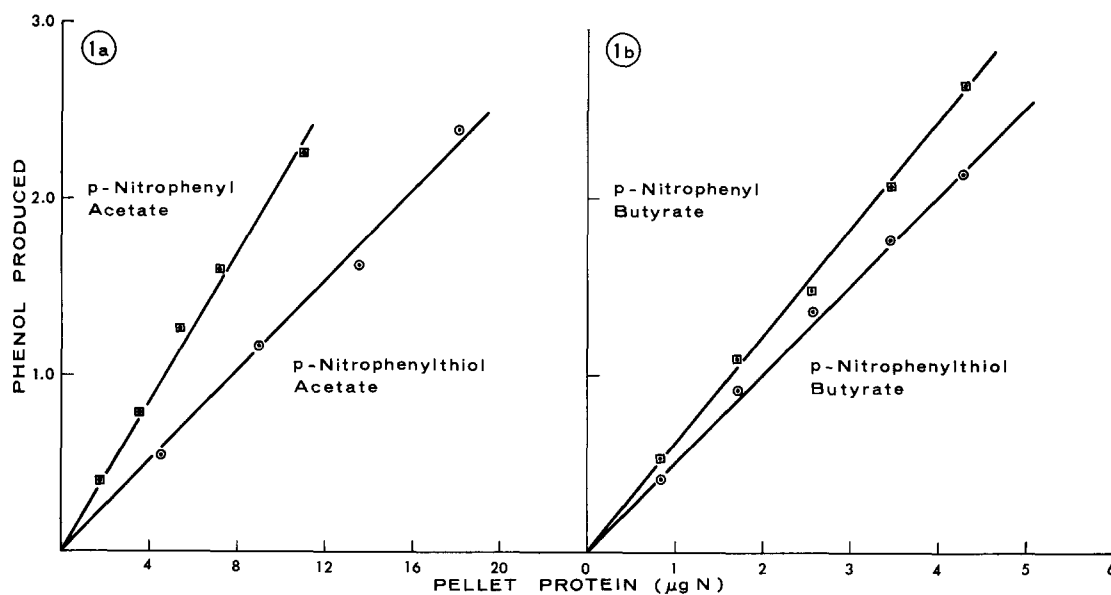
TABLE III
Distribution of Hydrolytic Activities in the Pellet and Supernatant Fractions of Lung

	Homogenate	Pellet fraction		Supernatant fraction	
	Specific activity*	Specific activity*	Total activity†	Specific activity*	Total activity†
			%		%
<i>p</i> -Nitrophenyl acetate	0.53	2.12	61	0.30	36
<i>p</i> -Nitrophenylthiol acetate	0.50	1.43	44	0.39	50
<i>p</i> -Nitrophenyl butyrate	1.36	4.33	48	0.65	30
<i>p</i> -Nitrophenylthiol butyrate	2.44	9.01	57	0.92	24

The reaction rates were determined in 2.7 ml 0.175 M sodium maleate pH 7.4 and 0.01–0.100 ml of enzyme in the sucrose-Tris medium. The reactions were initiated by the addition of 75 μ g of the esters in 0.30 ml water containing 10% acetonitrile. Rates were obtained at 410 m μ at room temperature (23°).

* The rate of the *p*-nitrophenol or *p*-nitrothiophenol production was calculated from the molar absorptivity at 410 m μ of *p*-nitrophenol or *p*-nitrothiophenol which was found to be 1.25×10^4 cm² mmole⁻¹ at pH 7.4 in the maleate or phosphate buffer. The units are μ moles *p*-nitrophenol or *p*-nitrothiophenol produced per mg N per min.

† The total activities were calculated as follows: Specific activity \times total mg N in fraction/total activities in homogenate \times 100. The homogenate contained 34.9 mg N, the pellet fraction 5.8 mg N, and the supernatant fraction 22.3 mg N; Recovery of N was 80.5%.



FIGURES 1 a and 1 b Relationship of phenol or thiophenol production to the amount of pellet protein. Ordinate, phenol or thiophenol production in micromoles per min $\times 10^2$; abscissa, pellet protein in micrograms N. Reaction rates were determined in 2.7 ml of 0.175 M sodium maleate buffer pH 7.4 and 0.01–0.15 ml of pellet suspension in the sucrose-Tris medium. The reactions were initiated by the addition of 75 μ g of the appropriate ester or thiol ester in 0.30 ml water containing 10% acetonitrile. Reaction rates were obtained at 410 m μ at room temp. (23°) and were calculated from the molar absorptivity of 1.25×10^4 cm² mmole⁻¹ determined for *p*-nitrophenol or *p*-nitrothiophenol under these conditions.

centration at constant substrate concentration for the four substrates tested.

pH EFFECTS: The rates of hydrolysis of PNPA and PNTA increase with increasing pH. Below pH 6.5 the rates of enzymatic hydrolysis are low, and above pH 8.2 in tris-maleate buffer the spontaneous hydrolysis of the thioesters is rapid. We selected the maleate buffer for all our studies because it appeared to be ideal for the cytochemical work. Since maleate loses its buffering capacity at pH values above 7.4, we chose this value. Sodium ethylenediaminetetraacetate (EDTA) or phosphate can substitute for maleate buffer for both electron microscopic and kinetic work.

INHIBITION: Table IV lists the action of the inhibitors tested. The differential inhibition shown by DFP on the supernatant and the pellet fraction suggests different enzymes. PMSF acts like DFP.

TABLE IV
*Effect of Inhibitors of Hydrolytic Enzyme
on Lung Fractions*

Inhibitor	Concentration	Activity remaining in fraction*	
		Supernatant fraction	Pellet fraction
	<i>M</i>	%	%
None		100	100
Diisopropyl phosphorofluoridate	10^{-9}	100	83
Same	10^{-8}	100	41
Same	10^{-6}	92	13
Phenyl methylsulfonyl fluoride	10^{-6}	94	18
Eserine sulfate	10^{-3}	93	84
Amberonium chloride	10^{-3}	100	100
BW-284	10^{-5}	100	100
Same	10^{-3}	88	100
KCN	10^{-3}	94	89

Reaction rates were determined in 2.7 ml 0.175 M sodium maleate pH 7.4 and 0.010-0.100 ml of enzyme suspension in sucrose-Tris medium. Inhibitors were added 5 min before the reaction was initiated by the addition of 75 μ g of *p*-nitrothiophenyl acetate in 0.3 ml of water containing 10% acetonitrile. All operations were carried out at room temperature (23°).

* Reaction rates were evaluated in terms of the absorbance changes at 410 m μ , and the rate of the reaction without addition of inhibitor was taken as 100% activity.

Eserine sulfate, ambenonium chloride, BW 284, and KCN do not inhibit.

Table V shows the effects of reagents used for electron microscopy on the reaction in vitro with lung homogenates. Formaldehyde and DMSO do not affect the reaction, whereas glutaraldehyde inhibits it. The results of the inhibition studies correlate with the results of the cytochemical work.

Fig. 2 shows the heat inactivation of the pellet enzymes toward the substrates. The hydrolytic activity is decreased to less than 50% after heating

TABLE V
*Effect of Reagents Used for Preparation of Specimens
on the Hydrolysis of *p*-Nitrophenylthiol Acetate
by the Lung Pellet Fractions*

Reagent	Conc.	Time of preincubation	Activity of control*
		% min	%
None	—	—	100
Formaldehyde†	4.0	5	95
Same	4.0	60	78
Glutaraldehyde	4.0	5	25
Same	4.0	60	23
Dimethyl sulfoxide	1.7	10	111
Same	6.7	10	100
Same	8.4	10	104
Same	10.0	10	77

The effect of the various compounds was tested as follows: 2.7 ml of sodium maleate pH 7.4 and 0.010-0.100 ml of enzyme suspension in the sucrose-Tris medium were placed into a cuvette together with the test compound. After the indicated preincubation time, the reaction was started by the addition of 75 μ g of *p*-nitrophenylthiol acetate in 0.3 ml water containing 10% acetonitrile. When dimethyl sulfoxide was used, the volume of the buffer was decreased to compensate for the volume change. All operations were carried out at room temperature (23°). Replacement of the sodium maleate buffer with 0.10 M sodium EDTA pH 7.5 gave identical control rates.

* Reaction rates were evaluated by the absorbance changes at 410 m μ , and the control rate was taken as 100%.

† The formaldehyde-buffer solution was prepared fresh by placing paraformaldehyde into the sodium maleate buffer and heating until the solid was in solution. Glutaraldehyde (50% solution, Biological Grade; Fisher Scientific Co., St. Louis, Missouri) was added to the buffer.

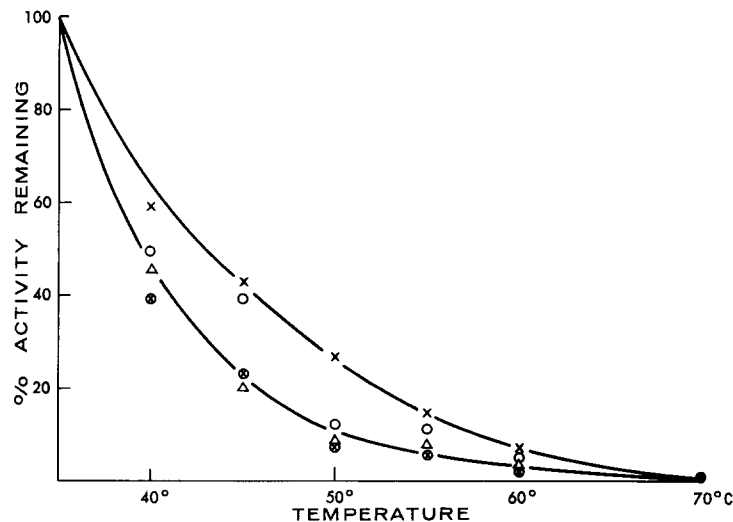


FIGURE 2 Heat inactivation of the hydrolytic activity of the pellet protein. Ordinate, per cent activity remaining after treatment. Activity at 23° equals 100%. Pellet protein was incubated in 2.7 ml of 0.175 M sodium maleate pH 7.4 for 10 min at the indicated temperature. After the sample had been brought to room temperature in a water bath, the reaction was initiated by the addition of 0.30 ml of water containing 75 μ g of the appropriate ester or thiol ester and 10% acetonitrile. The following substrates were used: ○—○, *p*-nitrophenyl acetate; ×—×, *p*-nitrophenylthiol acetate; △—△, *p*-nitrophenyl butyrate; ⊗—⊗, *p*-nitrophenylthiol butyrate. Reaction rates were obtained at 410 m μ at room temp. (23°) and were calculated from the molar absorptivity of 1.25×10^4 cm² mmole⁻¹ determined for *p*-nitrophenol and *p*-nitrothiophenol under these conditions.

for 10 min at 40°C and is totally destroyed at 70°C. The spontaneous hydrolysis of thiol esters in the presence of heat-killed enzyme is negligible. This sensitivity confirms the enzymatic nature of the reaction.

Cytochemical Observations

In the thin sections of tissue, the hydrolysis of the thiol esters yields PNT which then reacts with the gold ions present to yield an insoluble deposit that clearly delineates the site of enzymatic action. The deposit (Fig. 3) may appear within the cells as massive accumulations, or it may be found as fine filaments or granules (Fig. 4). The different substrates we have used do not affect the forms of the deposit. Thiolacetic acid incubated with gold gives a similar deposit. Granules less than 40 Å in diameter are seen either as individuals or in linear arrays apparently on filaments (Fig. 4). As the time of incubation is lengthened, these filaments increase in diameter and pack closer together, forming sheetlike aggregates. Occasionally the deposit may be so heavy that the internal structure of the organelle is

masked (Figs. 3, 5). Distribution of the deposit is not restricted to a single cell type.

The greatest amount of deposit, and hence activity, is found in the lamellar bodies of the giant alveolar cells (Figs. 3, 5). Lamellar bodies in the same cell may vary with respect to amount of deposit; some show almost no deposit, whereas others have an extremely dense deposit (Figs. 3, 5). Usually all lamellar bodies exhibit some product (Fig. 3). When tissues are incubated for as long as 1 hr, deposits may be found within the mitochondria especially on the inner membrane and cristae (Figs. 4, 6). In the giant alveolar cells, very few deposits are found associated with the Golgi complex or with other membrane systems. In cells in which such associated deposits occur, usually some of the mitochondria have reacted. Depositions associated with the nuclear envelope are rare, and the plasmalemma of these cells as well as the surfaces of the microvilli are free of reaction product.

Small multivesicular bodies, less than 0.5 μ , contain vesicles that appear identical with free vesicles, 0.05–0.1 μ , in the vicinity of the Golgi complex

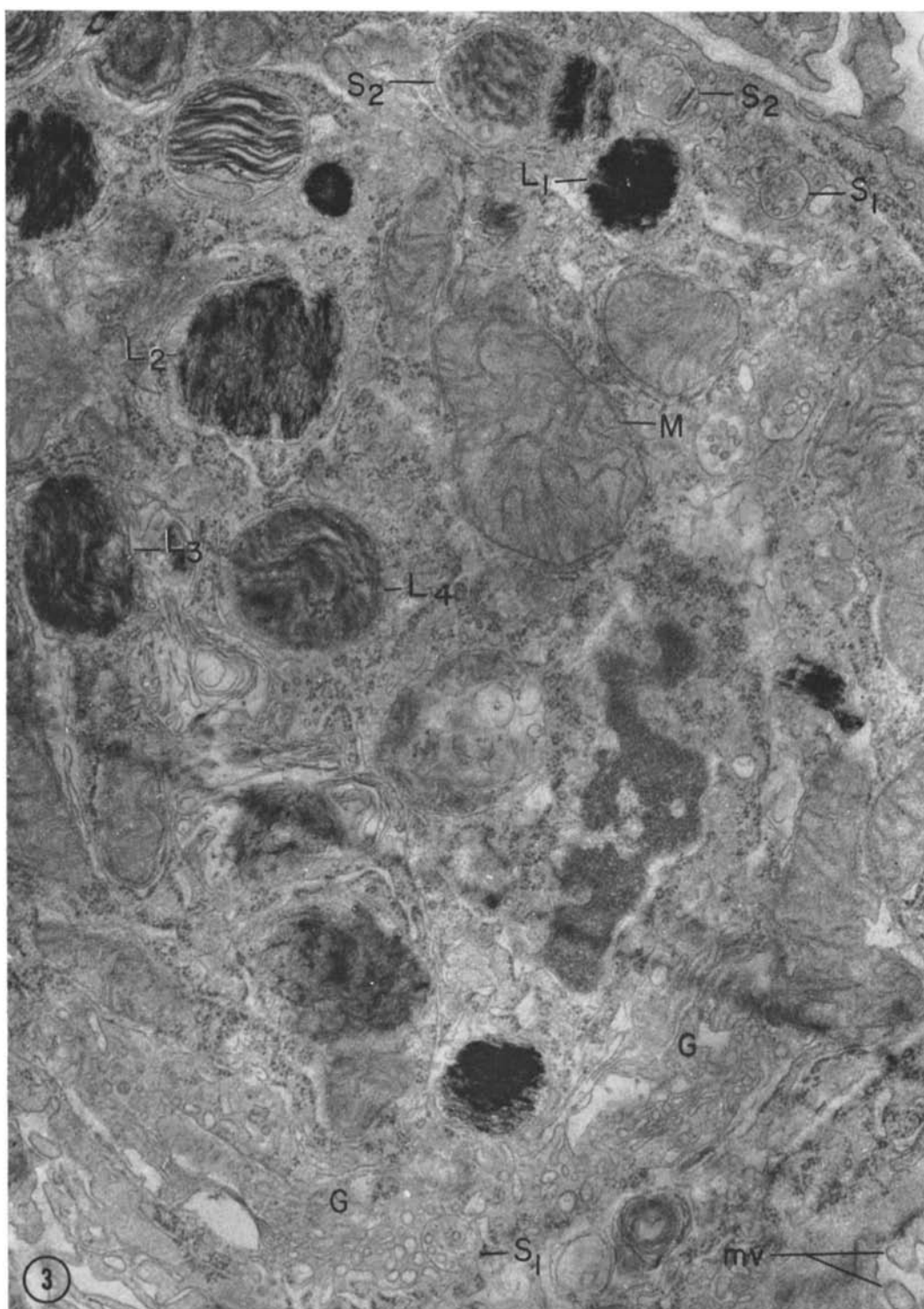


FIGURE 3 Portion of a giant alveolar cell showing details of the cytoplasm. The multilamellar bodies (L_1 - L_4) exhibit different amounts of an opaque reaction product resulting from the action of the enzyme on the substrate. The Golgi complexes (G), small multivesicular bodies (S_1), and mitochondria (M) are unstained. Organelles which appear to be transitional forms between the small vesicular bodies and the multilamellar bodies usually contain positively stained lamellae (S_2) along with unstained small vesicles. *mv*, microvilli. All specimens fixed and stained according to method outlined in text. Substrate, *p*-nitrophenylthiol acetate. $\times 34,000$.

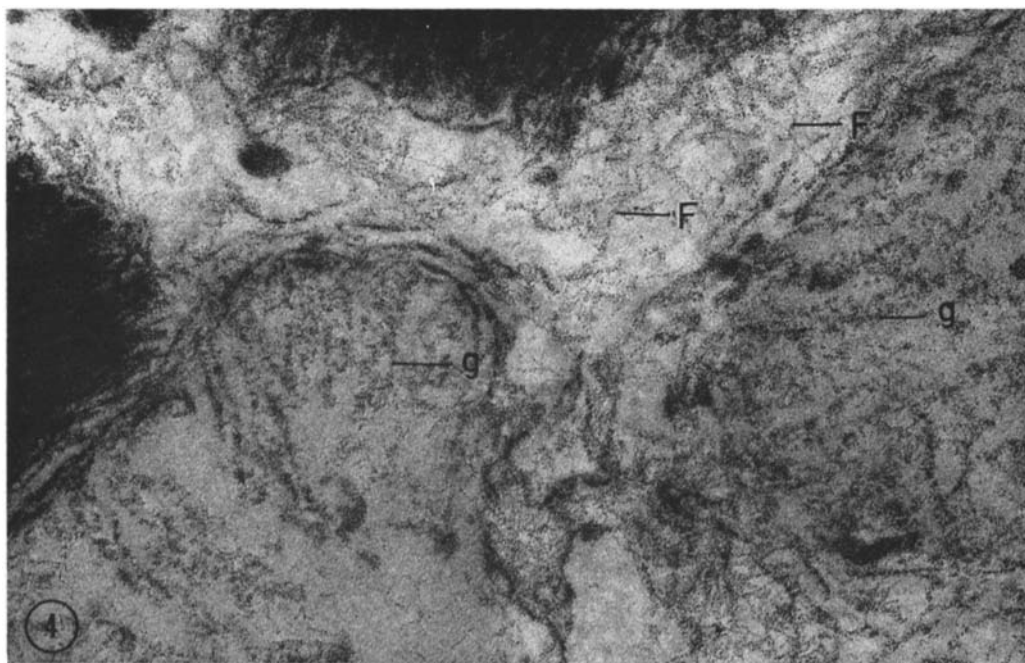


FIGURE 4 Details of portions of several lamellar bodies and mitochondria within a giant alveolar cell. Note the filamentous (*F*) arrangement of the cytochemical marker and its fine particulate nature. The mitochondria show small granules (*g*) of deposit along the membranes, including the cristae. Substrate, *p*-nitrophenylthiol acetate. $\times 143,000$.

(Fig. 3). No reaction product is found in either of these structures or in the cisternae of the Golgi complex. Larger multivesicular bodies, $0.5\text{--}1\ \mu$, (Fig. 3) may contain some reaction product which, if present, lies between the vesicles but within the limiting membrane of the larger body. Some multilamellar bodies are found which are crowded with lamellae exhibiting large amounts of reaction product but which contain a few small vesicles, $0.05\text{--}0.1\ \mu$, lacking any deposit.

The squamous epithelial cells lining alveoli and the endothelial cells exhibit similar patterns of product deposition, but the giant alveolar cell has a different pattern (Figs. 5, 6). In the giant cell, the cisternae composed of agranular membrane contain the deposits in irregular profiles of vesicles, tubules, and other membrane systems. Often adjacent cells exhibit differences in the distribution of product; the nuclear envelopes of adjacent cells may stain differently. Cells exhibiting positive reactions at the nuclear envelope usually contain large amounts of deposit in association with other cytoplasmic membranes, especially in the vesicles which appear to be portions

of the Golgi complex (Fig. 6). The degree of reaction associated with the Golgi complex of the different cells varies. The reaction in pinocytotic vesicles is also variable (Fig. 5); when it is positive, the closed vesicles may be filled with deposit, whereas the invaginations of forming or secreting vesicles sometimes contain a deposit on the extracellular face of the plasmalemma. All mitochondrial membranes stain (Figs. 4, 6); usually the deposit consists of fine granules scattered over the membranes, although it may be massive and fill the mitochondrion and the spaces between the outer and inner limiting membranes.

INHIBITORS: The inhibition pattern noted in the *in vitro* studies of the lung fractions is paralleled by the cytochemical observations. DFP inhibits the reaction after a short incubation time (Fig. 7); however, after longer incubation periods, even when fresh DFP solution was added every 15 min to the incubation mixture, a positive stain similar to that of the control occurred although less intense.

PMSF gives an inhibitory effect after a brief incubation period, although some deposit may be



FIGURE 5 Portion of a giant alveolar cell (C), squamous epithelial cells (Ep), and endothelial cells (En). The multilamellar bodies (L) vary in the amount of reaction product. The cytoplasm of the epithelial and endothelial cells contains numerous positively stained vesicles and some stained membrane forms. These cells also show many positively stained pinocytotic vesicles (p). Alveolar space, AS. Substrate, *p*-nitrophenylthiol acetate. $\times 26,000$.

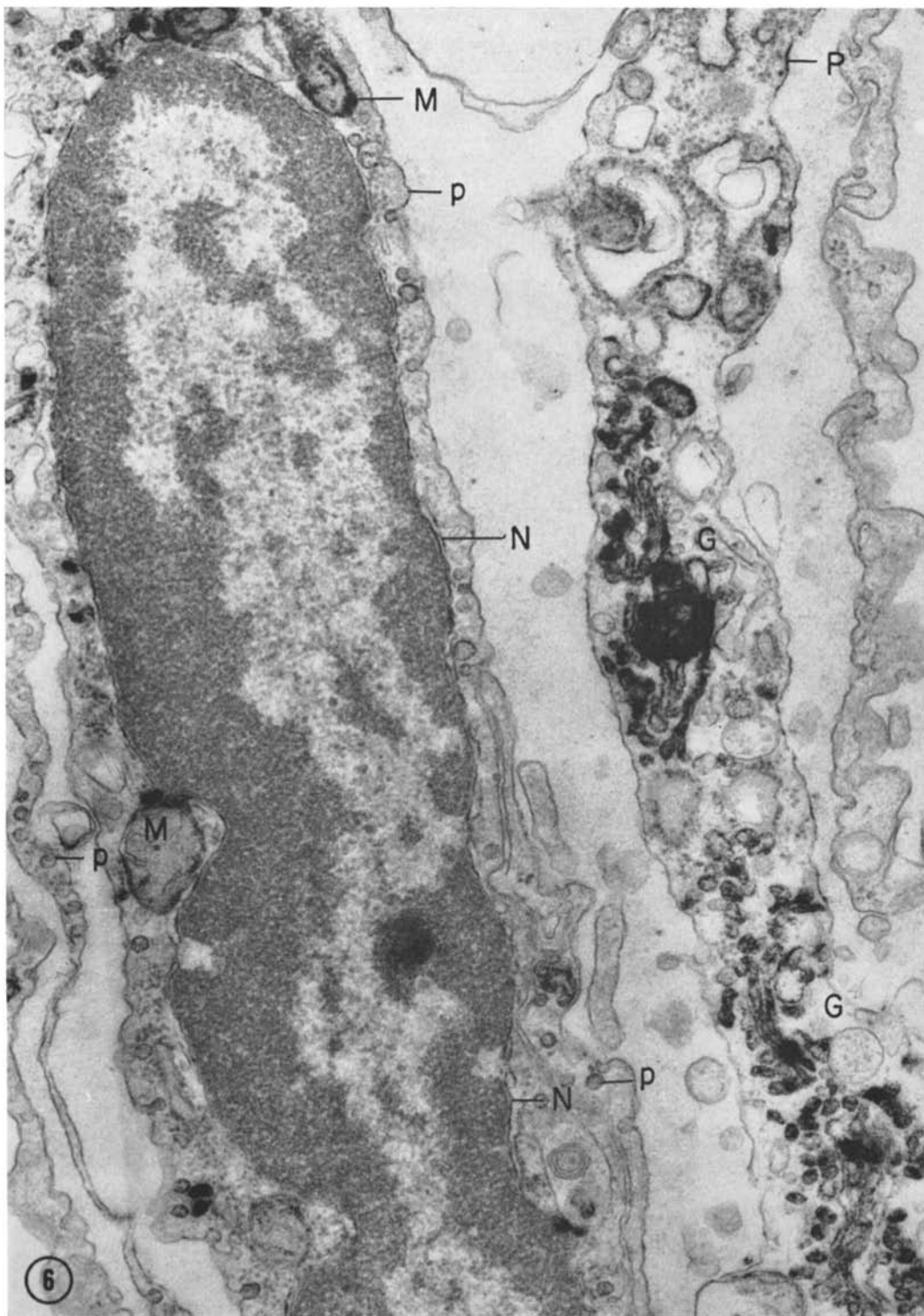
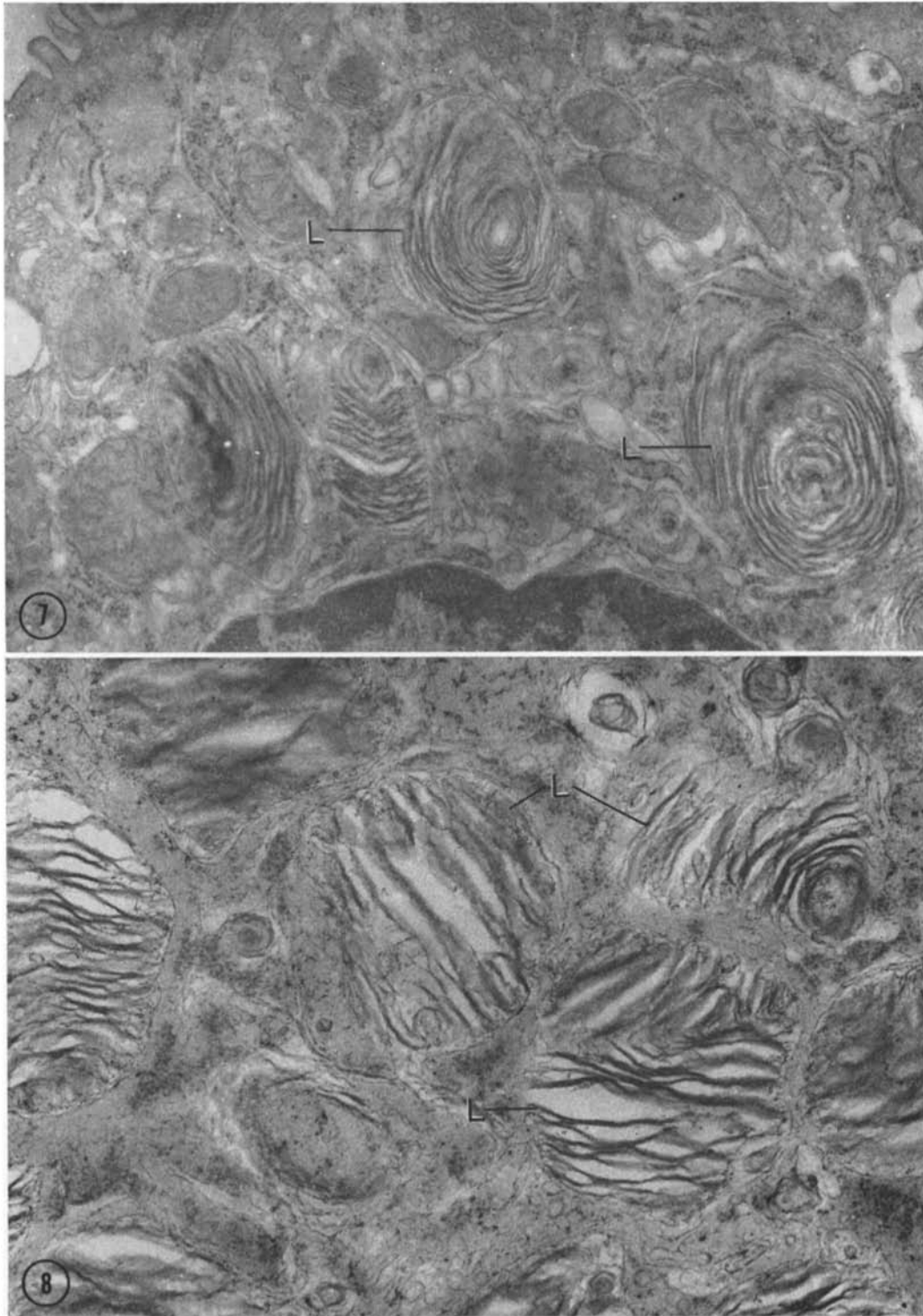


FIGURE 6 Portion of the cytoplasm of several endothelial cells showing positively stained Golgi complexes (*G*). Some reaction has taken place on the nuclear envelope (*N*), mitochondrial membranes (*M*), pinocytotic vesicles (*p*), and along portion of the cell membrane (*P*). Substrate, *p*-nitrophenylthiol butyrate. $\times 38,000$.



FIGURES 7 and 8 Portions of the specimens treated with DFP (Fig. 7) and PMSF (Fig. 8) exhibit few scattered granules of deposit after short (15 min) incubation time. These results correlate with the in vitro experiments. After longer incubation periods (45-60 min) some product does accumulate. *L*, multi-lamellar bodies. Substrate, *p*-nitrophenylthiol acetate. Fig. 7, $\times 25,000$; Fig. 8, $\times 35,000$.

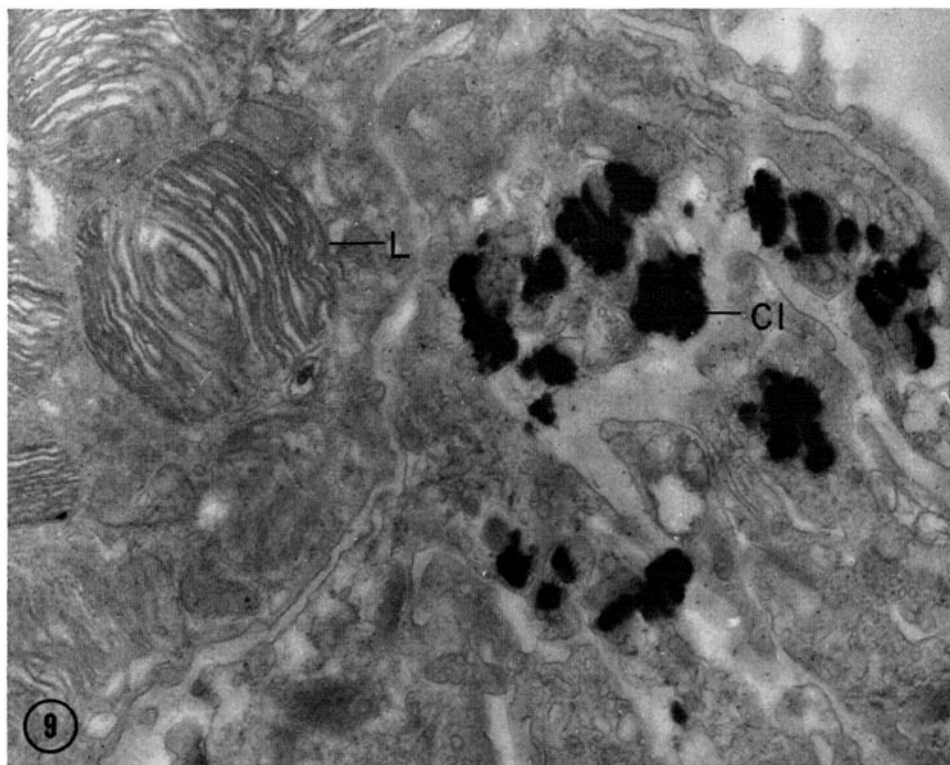


FIGURE 9 Specimen treated with BW 284. Random clumps of marker (*Cl*) are dispersed throughout the cytoplasm of the epithelial and endothelial cells without relation to any specific cellular structures. No deposit is present in the multilamellar bodies (*L*). Substrate, *p*-nitrophenylthiol acetate. $\times 28,500$.

seen scattered throughout the cytoplasm of cells, especially giant alveolar cells (Fig. 8). Specimens incubated for 60 min showed distinct deposition similar to that of the control, although it, too, was less dense. PMSF might be a satisfactory substitute for DFP. It is easily handled and much less toxic.

Eserine did not inhibit the cytochemical reaction. Ambenonium chloride also does not appear to inhibit it; however, close inspection reveals scattered granules of deposit in locations in which deposit is not found in control specimens. This scattering may be the result of redistribution of the enzyme. BW 284 (Fig. 9) did not prevent the formation of reaction product, but after incubation with this inhibitor the distribution of product is entirely different from that of the control. Random clumps of deposit unrelated to any specific cellular structures were found in squamous epithelial cells and endothelial cells, and no product was found in the giant alveolar cells. BW 284 does not inhibit the *in vitro* assay (Table IV).

Extraction of the Reaction Product

The reaction product was removed from reacted ultrathin sections mounted on carbon-coated grids by washing with pyridine for 30 sec. Fig. 10 shows the appearance of the section after removal of the deposit by the solvent.

In an attempt to identify the deposit, we carried larger amounts of cut-up tissue through the fixation and reaction procedures outlined above; after the cytochemical reaction was complete, we washed the bits thoroughly with ethanol and then extracted with pyridine. This pyridine extract was analyzed for gold in a Philips X-ray fluorescence spectrophotometer⁴ equipped with lithium fluoride optics and a molybdenum tube. Readings were taken at 50 kv and 40 ma. The fluorescence

⁴ We wish to thank Dr. Fred Leaver, Veterans Administration Hospital, Denver, Colorado and the Coors Spectro-Chemical Laboratory, Golden, Colorado for their kind cooperation in carrying out these analyses.

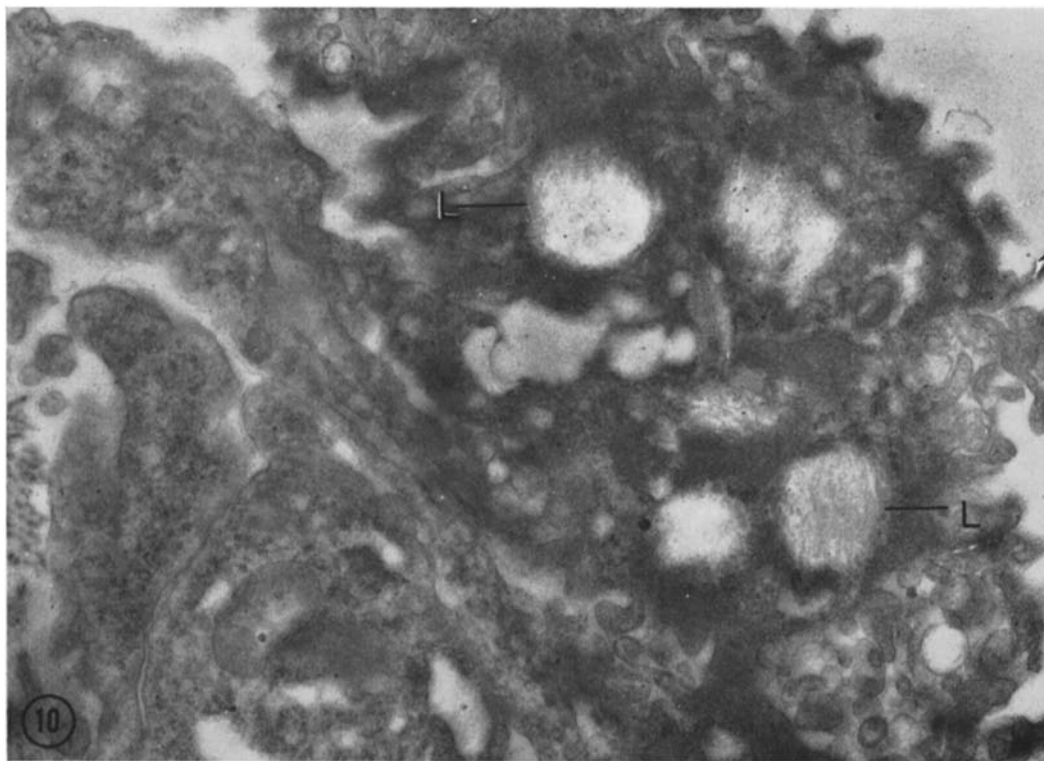


FIGURE 10 The deposits of the opaque reaction product can be extracted from tissues by pyridine. Removal of the marker from the multilamellar bodies and other positively stained organelles is shown. Substrate, *p*-nitrophenylthiol acetate. *L*, multilamellar bodies. $\times 18,000$.

wavelength and the pulse height of the fluorescence were identical to those observed with gold foil. Other aliquots were examined for gold content by emission spectrometry.⁴ One rabbit lung (1.8 g of protein) contained 140 μg gold.

The absorption spectrum of the pyridine extract described above was examined and compared with the spectrum of the known gold *p*-nitrothiolphenolate in pyridine solutions. The known compound has a distinct peak at 405 $m\mu$. The tissue extract has a shoulder of absorbance at this wavelength.

DISCUSSION

Enzymes are present in lung that hydrolyze the acetate, propionate, and butyrate esters of *p*-nitrothiophenol. The product of hydrolysis of *p*-nitrophenylthiol acetate was identified as *p*-nitrothiophenol. The octanoyl ester was too insoluble to be tested.

Cytochemical End Product

When *p*-nitrothiophenol is treated with aurous ions in vitro, the product is aurous *p*-nitrothiolphenolate (as detailed under Materials and Methods). The extracted cytochemical product has solubility and spectral properties compatible with those of the synthetic aurous *p*-nitrothiolphenolate.

Inhibitors

DFP: The inhibition of esterase by DFP in the cytochemical reaction needs some comment. After short-time incubation (15 min) in the presence of DFP the reaction is inhibited, but after incubation for an hour a definite reaction occurs. A possible explanation of this phenomenon is the presence in the tissue of a relatively small amount of DFP-insensitive enzyme which, with longer incubation periods, can make sufficient product for visualization. This explanation is supported by the presence of DFP-insensitive enzyme in the supernatant fraction in the in vitro studies described. A por-

tion of the pellet enzymes is also DFP-insensitive (10–15%).

BW 284, considered a specific inhibitor of acetyl cholinesterase because of its quaternary ammonium structure, does not inhibit the activity of the homogenate. The cytochemical observations indicate apparent release and dispersion of the enzyme from the lamellar bodies after treatment with this inhibitor. The quaternary ammonium group has surface-active properties which might affect membrane permeability and thus account for this observation. In examining this possibility, we tested the action of a surface-active agent, Triton WR 1339 which does not have a quaternary ammonium group, on the cytochemical reaction and found similar dispersion of product. This finding indicates that the lamellar bodies are labile (see below). To check whether some of these dispersions might be the result of immediate postmortem changes in the lungs, we prepared some specimens by injecting incubation mixture, with and without fixative, intratracheally into the still living lung. These specimens showed exactly the same distribution of the cytochemical product as those tissues prepared in the usual way.

Substrate Specificity

Since the lung homogenate does not hydrolyze acetyl choline and since the cytochemical reaction is not inhibited by eserine, the presence of acetyl choline hydrolase (3.1.1.7) and acylcholine acyl-hydrolase (3.1.1.8) can be ruled out. The lung homogenate described in this paper hydrolyzes *N*-benzoyl-*L*-tyrosine *p*-nitroanilide, a specific substrate for chymotrypsin, a peptidyl peptide hydrolase (3.4) (34). Chymotrypsin can hydrolyze PNTA (Reiss, O. K. Unpublished observations.) as well as PNPA. The lung also possesses glycerol-ester hydrolase (3.1.1.3) (35), a class of enzymes which can hydrolyze PNTA (Reiss, O. K. Unpublished observations). Homogenates of liver can hydrolyze thiol esters (36).

The presence of weak cytochemical reactions on the mitochondrial and nuclear membranes is not understood at this time. The functional significance of the described enzymatic activity of the lung must await further investigation.

Lamellar Bodies and Pulmonary Surfactant

Most of the esterase activity toward the substrates dealt with in this study is found in the

lamellar bodies. Although the pellet fraction of the centrifuged homogenate of adult rabbit lung contains much of this esterase activity, which is DFP-sensitive, our search for the lamellar bodies in fixed pellet specimens with electron microscopy has revealed very few of these organelles. This observation agrees with our previous report (1) that the organelles are extremely labile and rupture during homogenization and thus leave some of the enzymatic protein attached to the sedimented membranes. We have found other indications that this assumption is correct; the enzymatic activity of the pellet fraction toward the thiol esters becomes further solubilized after it has stood a few days in the cold or after treatment with sonic oscillation.

The giant alveolar cell has long been presumed to be secretory, and recent work has suggested that it may be a source of the important pulmonary surfactant (37, 38). This surfactant presumably functions by lowering surface tension and thus stabilizes the alveoli for efficient respiratory gas exchange. It is now believed to be a lipoprotein (39, 40) which contains large amounts of dipalmitoyl lecithin. Previous work (41) indicates that surfactant activity is present in the pellet fraction described in this paper. Partially purified surfactant fractions obtained from throat washings by the procedure of Bondurant and Miller (42) can hydrolyze PNTA. This finding suggests that the esterase and surfactant activities may be inter-related.

The electron micrographs support the idea that the lamellar bodies are formed from the multivesicular bodies and that they develop their esterase, and possibly surfactant, activity (43) and discharge this activity into the alveolar lumen (38, 44). The electron micrographs show a complete gradation between small and large multivesicular bodies and partially and completely lamellated bodies. Some of the lamellar bodies stain deeply, some not at all, and some at intermediate levels, as would be anticipated in a sequence of development, activation, and release of a secretory granule. Similar morphological sequences have been shown by Caro and Palade (45) for the secretory granules of the acinar cell of the pancreas and by Smith and Farquhar (46) for granules of the pituitary cells. The lamellar bodies, like the zymogen and pituitary granules, lose their structural integrity when released from the cell.

When one considers the nature of the lamellar bodies, the question arises whether they can be classified as lysosomes. The lamellar bodies give a weak cytochemical reaction for acid phosphatase, whereas the lysosomes of the alveolar macrophages give an intense reaction. Conversely, the esterase activity of the lamellar bodies is strong but that of the macrophage is weak. The enzymes of the lysosomes are most active in the acid region, however the esterases described here function in the neutral or alkaline regions. For these reasons, we favor the interpretation that the lamellar body is a secretory organelle rather than a lysosome.

The cytochemical method outlined here may prove useful in other connections where it is possible to design synthetic substrates which can

form mercaptides after enzymatic reaction. The insolubility and small size of the precipitate of the mercaptide makes it an ideal cytochemical product. Furthermore, the removal of these precipitates by specific solvents permits their chemical identification.

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