RADIOAUTOGRAPHY OF CHOLESTEROL IN LUNG

An Assessment of Different Tissue Processing Techniques

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ABSTRACT

30 Swiss albino mice aged 8 days were injected intraperitoneally with 0.2 ml of a solution of 4% N,N-dimethyl-formamide in 5% dextrose in water containing cholesterol-1,2-^{3}H (∼1 mCi/ml). Lung tissue was embedded in an Epon mixture after either acetone and propylene oxide dehydration, partial ethanol and Epon 812 dehydration, or the precipitation of cholesterol by digitonin succeeded by partial dehydration. The distribution of cholesterol-1,2-^{3}H in lung parenchyma in 1μ Epon section radioautograms was compared with that in frozen section radioautograms and was found to be independent of the manner of tissue processing. Grain distribution in the tissue was essentially the same whether 16, 63, 93, or 100% radioactivity was retained in the lung. However, grain distribution in the alveolar spaces differed, presumably due to displacement of pulmonary surfactant, which contains cholesterol. Intracellular distribution of cholesterol, in electron microscope radioautograms, was the same with either 51% or 93% retention of radioactivity in the lung. Loss of radioactivity into the various processing solutions was monitored. The various processing techniques have different drawbacks.

INTRODUCTION

An important current controversy in high resolution radioautography centers around the validity of radioautographic studies which employ soluble labeled compounds (29). Radioautography of labeled steroids has shown that massive translocation and loss of labeled compound occurs during histological processing of the tissues (39). Lipids are soluble in dehydrating agents and also in embedding plastics (26). Thus it is claimed that the only valid techniques for soluble compounds are those which use unfixed frozen or freeze-dried frozen sections where the sections do not come into contact with liquid solutions before development of the radioautogram (1, 11, 29, 37, 38, 39, 41). These techniques are currently only well established at the light microscope level. Various tissues and labeled compounds have different characteristics which may determine whether redistribution of the soluble compound occurs in a tissue during radioautographic processing. For instance, Cope and Williams (7) found that no redistribution of neutral lipid occurred when peritoneal macrophages were processed for radioautography by 10 different methods in contrast to the situation found with steroids in the uterus (39).

Electron microscope observations of the distribution of cholesterol in tissues have frequently relied on the assumption that when cholesterol is precipitated by digitonin the precipitate remains

firmly bound to the tissue *in situ* during subsequent tissue processing (10, 43). Analyses of radioautographs after different tissue processing methods may help to elucidate the validity of this assumption.

Cholesterol has been shown to be metabolically stable in the lung (6, 20). If cholesterol is labeled with ^{3}H in the A ring, all the isotope is still attached to cholesterol after tissue processing (12). Thus the silver grains seen on radioautograms of lung after administration of cholesterol-1,2-^{3}H will localize the cholesterol.

Accordingly the aims of this study are to investigate the distribution of cholesterol-1,2-^{3}H in lung and to determine the extent of redistribution and loss of radioactivity which occurs during the routine histological processing which precedes radioautography.

Pulmonary surfactant contains approximately 7% cholesterol (9, 16, 23). The surfactant is displaced from the alveolar surface by the introduction of fixative solutions into the lung via the trachea (42). The localization of cholesterol in surfactant therefore poses a special problem which is also considered.

MATERIALS AND METHODS

30 Swiss albino mice aged 8 days were injected intraperitoneally with 0.16–0.25 ml of a solution of 4% *N*, *N*-dimethyl formamide (DMF)[1] in 5% dextrose in water containing cholesterol-1,2-[3]H ($\sim$1 mCi/ml) (New England Nuclear Corporation, Boston, Mass.), 30–50 Ci/mM (13). For these experiments the aqueous solution was made up by New England Nuclear Corporation and sealed under vacuum. Four other mice were injected with 0.2 ml of a solution of 4% DMF in 5% dextrose in water. All mice were sacrificed with intraperitoneal pentobarbital (0.05 µg).

Eight mice were used for Epon-embedded tissue radioautography. One mouse was sacrificed at 20 hr after injection, four at 48 hr, and three at 4 days after injection. One nonradioactive mouse was sacrificed with each group of radioactive mice. The trachea was exposed, cannulated, and the lung was fixed as quickly as possible by slow infusion of 5% cacodylate-buffered glutaraldehyde solution via the trachea until the lung filled the chest cavity (approximately ½ maximum lung volume). After approximately 30 min, small pieces of pulmonary tissue

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[1] *Abbreviations used in this paper:* DMF, *N*, *N*-dimethyl formamide; HD, half distance; NCS, Nuclear-Chicago solubilizing solution; POPOP; 1,4-bis[2-(5-phenyloxazoyl)]-benzene; PPO, 2,5-diphenyloxazole.

($\sim$1 mm[3]) from randomly chosen areas were transferred to fresh 5% buffered glutaraldehyde solution for a further 30 min of fixation. Tissues from each animal were processed for radioautography by the three methods outlined below.

Complete Dehydration (Acetone)

After 1 hr of glutaraldehyde fixation, tissues were transferred to Sabatini's washing solution and left overnight at 4°C (30). The lung tissue from the mouse sacrificed 20 hr after injection was postfixed for 1 hr in Dalton's chrome osmium (8). Tissues from the other seven mice were postfixed for 24 hr in Dalton's chrome osmium. All tissues were soaked for 1 hr in 1% uranyl acetate in 10% formalin (40), dehydrated in graded acetones (15 min each), infiltrated with propylene oxide, and embedded in an Epon mixture (19).

Partial Dehydration with Alcohol and Epon 812 (35)

Tissues were fixed and postfixed as described in the section on complete dehydration. Dehydration was carried out with two 5 min changes of 70% alcohol at 0°C, two 5 min changes of 95% alcohol at 0°C, and three 1 hr changes of Epon 812 at 0°C (35). Tissues were infiltrated overnight in an Epon mixture at 4°C, transferred to fresh Epon mixture, shaken and warmed to 37°C, and finally embedded after 90 min in the Epon mixture (19).

Digitonin Treatment

After 1 hr of fixation in 5% buffered glutaraldehyde, tissues were transferred to a 2.5% solution of glutaraldehyde with 0.2% digitonin and buffered with sodium cacodylate (final pH, 7.2) for 21 hr at 4°C (25). After rinsing with Sabatini's solution, the tissues were postfixed as described in the section on complete dehydration. Dehydration, infiltration, and embedding were carried out by the method outlined in the section on partial dehydration.

An additional 18 mice were sacrificed at time intervals varying from 1 to 35 days after injection, and the lungs were used to determine uptake of radioactive cholesterol into the lung and loss of radioactivity during tissue processing. The remaining four mice and one nonradioactive mouse were used for the frozen section radioautography.

For light microscope radioautography, sections of Epon-embedded tissue (1 µ thick) were cut on a Sorvall MT-2 microtome. Radioactive and control sections were mounted side-by-side on glass slides which were warmed to flatten and attach the sections. The sections were vacuum coated with a thin

layer of carbon and then coated with Kodak NTB-2 emulsion by the method of Kopriwa and Leblond (17). The slides were exposed in plastic boxes at 4°C and kept dry with silica gel. One slide in each batch was fogged by exposure to light and then stored with the others to check for latent image fading. For one experiment, half of the sections from one tissue block were not vacuum coated with carbon prior to coating with emulsion. Exposure lasted for 1–4 wk. In each batch of slides sections from one block were exposed for different time periods to determine whether the relationship between increasing grain density and increasing exposure time was linear. The slides were developed in Dektol (1/1) for 2 min at 15°C, stopped in 3% acetic acid, and fixed in Kodak Acid Fixer for 2 min. After rinsing in three 5 min changes of distilled water, the slides were air dried and stained with methylene blue (1%) in sodium borate (1%) for 30 sec at 40°C. After rinsing in distilled water and air drying, the sections were mounted in the Epon mixture (19). Photomicrographs taken before and after staining showed no evidence of translocation of grains during staining. Photomicrographs were obtained at an initial magnification of 666 with a Leitz microscope, a Nikon camera, and Panatomic-X film. The photographs were enlarged to × 4000 for analysis.

For electron microscope radioautography, pale gold sections (1000 A) were cut from Epon-embedded tissue on a Sorvall MT-2 microtome and placed on slides that had been coated with a layer of collodion (0.5% in amyl acetate). They were vacuum coated with a thin carbon layer and then covered with a monolayer of Ilford L4 emulsion (31). They were exposed at 4°C in plastic boxes containing silica gel for 4–6 months. The slides were brought to room temperature and developed in Microdol X (3 min at 24°C) (31). Electron micrographs were obtained with a Philips 200 electron microscope. Micrographs were enlarged to a final magnification of 14,000.

Uptake and Loss of Cholesterol

18 radioactive mouse lungs were used to measure the uptake of radioactivity into the lung and to monitor the loss of radioactivity which occurred in the tissue processing fluids. Three pieces of each lung (~20 mg) were weighed, digested in Nuclear-Chicago solubilizing solution (NCS) (Nuclear-Chicago Corporation, Des Plaines, Ill.), and counted in a scintillation fluor containing 2,5-diphenyloxazole (PPO) (4 g/liter) and 1,4-bis [2-(5-phenyloxazolyl)]-benzene (POPOP) (0.5 g/liter) in toluene. Tritium assays were carried out on a Packard Tri-Carb liquid scintillation system (3000 series). Quench correction was determined by external standardization. The activity of the tissue was expressed in dpm per milligram tissue wet weight.

Portions of each lung (~3 mg) were weighed, and processed by seven different processing techniques:

(a) Acetone dehydration and propylene oxide infiltration after 1 hr in Dalton's chrome osmium.

(b) Acetone dehydration and propylene oxide infiltration after 24 hr in Dalton's chrome osmium.

(c) Partial dehydration (ethanol and Epon 812) (35).

(d) Digitonin treatment (21 hr soak in digitonin followed by partial dehydration with ethanol and Epon 812).

(e) Digitonin treatment (21 hr soak in digitonin, acetone dehydration, and propylene oxide infiltration) (25).

(f) Digitonin treatment (40 hr soak in digitonin, acetone dehydration, and propylene oxide infiltration).

(g) Water-soluble embedding media: tissues were fixed and postfixed as described in the section on complete dehydration. Dehydration was carried out in increasing concentrations of Durcupan in water (Fluka AG, Buchs SG, Switzerland). Tissues were then infiltrated with 100% Durcupan (26).

The glutaraldehyde, Sabatini washing solutions, and samples of Dalton's chrome osmium solution were counted in Bray's solution (3). The dehydrating and infiltrating solutions were counted in toluene-based scintillation fluor after evaporation of volatile materials. The dehydrated and infiltrated tissues and solutions containing Durcupan were digested in NCS solution and counted in the toluene-based scintillation fluor.

Identity of the cholesterol-1,2-³H in mouse lung before and after histological processing was determined by extraction of the fresh and fixed lung tissues with chloroform:methanol with subsequent thin layer chromatography and liquid scintillation counting of the fractions derived from the thin layer plate. 94% of the radioactivity in the tissue prior to processing was recovered in the chloroform:methanol extract, tissue residue, and saline phase. The saline phase accounted for less than 0.1%. 92% of the radioactivity recovered from the thin layer plate was located in the cholesterol band. After dehydration and infiltration, 95% of total counts from thin layer chromatograms was in the cholesterol band. This fraction was confirmed as cholesterol by comparing retention time of the acetylated derivative with that of a cholesterol acetate standard in a gas chromatograph. The loss of radioactivity into pooled fixative solutions and washing solutions was consistently less than 1% of the recovered radioactivity.

Frozen Section Radioautography

For frozen section radioautography two mice were sacrificed at 2 days, one at 5 days, and one at 15 days after injection of cholesterol-1,2-³H. A nonradio-

active mouse was sacrificed 4 hr after injection of the carrying solution. The lungs were inflated to $\frac{1}{2}$ maximum lung volume with air, and the trachea was ligated. The upper lobes from the right and left lungs were ligated and placed still inflated on a liver base-plate attached to a cryostat tissue holder. The lobes on tissue holders were immersed in isopentane at the temperature of liquid nitrogen ($-196\,°C$). After 1–2 min, they were transferred to liquid nitrogen and stored until they were cut; this was a maximum time of 2 hr. Sections (2–4 μ) were cut on a CTD Harris-International microtome cryostat at a temperature of $-30\,°C$ and picked up onto emulsion-coated cover slips at a temperature of approximately $-10\,°C$, using a safelight (1). The cover slips had been mounted with Permount on slides to facilitate handling, coated with Kodak NTB-2 emulsion (17), and stored in the cryostat in the presence of silica gel. The sections were not allowed to thaw. They were exposed at $-85\,°C$ in the presence of silica gel. Control sections and radioactive sections on light fogged slides were exposed under the same conditions to check for negative chemography as described by Rogers (27, 28). After an exposure time of 1–4 wk, slides were brought to the temperature of the cryostat, fixed with a drop of Wohlman's fixative for 1 min, rinsed at room temperature in four 1 min changes of distilled water, and developed with Dektol (1/1) as described. Sections were also fixed in formaldehyde vapor to test for section dislocation. Sections were stained either with hematoxylin and eosin or with methylene blue (1%) in sodium borate (1%). Cover slips were removed with a drop of xylene, inverted, and mounted in Permount. Photomicrographs were taken as described for Epon section radioautograms.

The light microscope radioautograms were analyzed as follows: Photomicrographs of randomly selected areas of radioautograms prepared from Epon-embedded sections and frozen sections were obtained at three different focal depths. A lattice with points spaced 1 cm apart was superimposed on the photomicrographs in order to give a measure of the relative area occupied by each tissue compartment (5).

ANALYSIS I: Grains and points in frozen sections and in Epon sections were tabulated over blood vessels in areas more than 1 μ away from the endothelium, over areas of the alveolar lumen more than 1 μ away from the alveolar wall, and over random areas of the alveolar wall. 1 μ is approximately twice the expected resolution in light microscope tritium radioautograms (13). The grain density, number of grains divided by the number of points, was calculated for all three compartments. The standard deviation for each compartment was estimated from the formula $G/P \sqrt{(1/G)^2 + (1/P)^2}$ where G = number of grains and P = number of points (13).

The grain density found over areas of the slide away from the section was considered to be background activity and was subtracted from each grain density. Grain densities were compared by conversion of the grain density over the alveolar wall compartment to unity; the densities of the other two compartments were then multiplied by the same factor.

ANALYSIS II: The photomicrographs of Epon-embedded sections were divided into the following compartments: 1, alveolar lumen, 2, epithelial edge, 3, capillary lumen, 4, nucleus, 5, cytoplasm. Grains and points were counted over each of these compartments. Alveolar macrophages were excluded from the counts over the alveolar lumen. The epithelial edge included only grains and points on the edge of the tissue. The capillary lumen compartment included erythrocytes, granulocytes, and plasma and excluded endothelium. The nucleus compartment included nuclei of all epithelial cells, septal cells in the alveolar walls, and endothelial cells. The cytoplasmic compartment included the capillary endothelium, the interstitial fluid, and the cytoplasm of all cells making up alveolar walls. Grain densities were calculated for each compartment and compared by conversion of the grain density in the cytoplasmic compartment to unity; the grain densities of the other compartments were then multiplied by the same factor.

ANALYSIS III: The alveolar lumen and surrounding tissue in both frozen sections and Epon sections were divided into the following compartments:

A. Edge of alveolar wall. This compartment was 0.66 μ wide. This distance was arbitrarily chosen because of the indistinctness of the frozen section radioautograms. The tissue edge when visible was the midpoint of this distance.

B. Tissue. 1 μ into the tissue from the tissue border of compartment A.

C. 0–0.5 μ from the lumen border of compartment A into the alveolar lumen.

D. 0.5–1.0 μ into the alveolar lumen from the lumen border of compartment A.

E. 1.0–1.5 μ into the alveolar lumen from the lumen border of compartment A.

F. 1.5–2 μ into the alveolar lumen from the lumen border of compartment A.

G. Alveolar lumen, more than 2 μ away from an alveolar wall.

Compartments E and F were only counted in photomicrographs of radioautograms prepared from frozen sections taken from animals sacrificed 2 and 5 days after injection. For all other radioautograms grains and points in these compartments were counted as part of the alveolar lumen (compartment G). Grain densities were compared after conversion of the edge compartment (A) value to unity and multiplication of the other densities by the same factor. All distances were converted to half distance
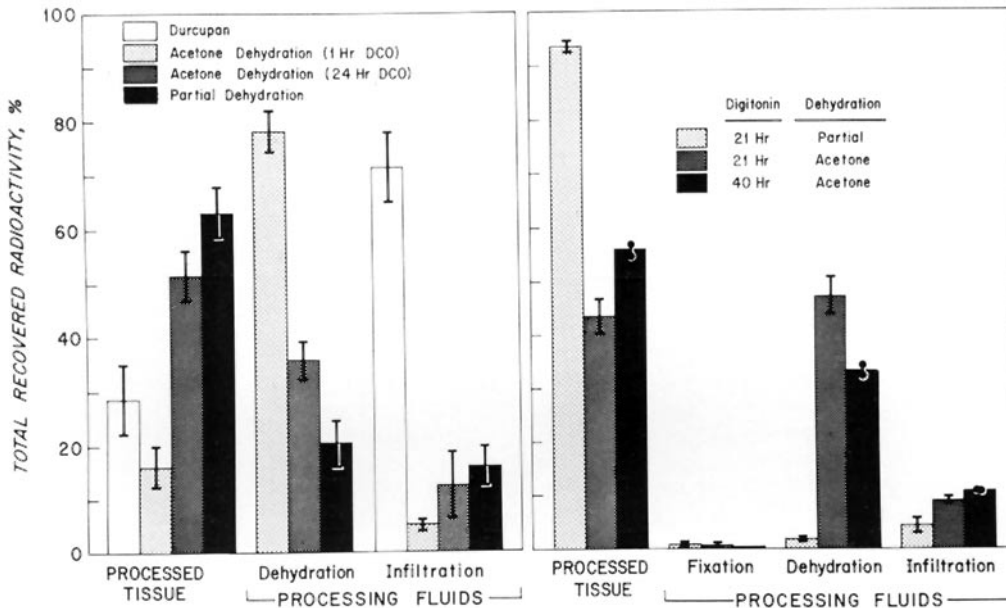
FIGURE 1 The fate of cholesterol-1,2-^{3}H in mouse lung during processing for electron microscopy. The radioactivity recovered from the processed tissue, the dehydrating solutions, and the infiltrating solutions is taken as 100%. Acetone dehydration followed by Epon embedding retains 16% of the radioactivity in the tissue; this is increased to 51% when postfixation with osmium is increased from 1 to 24 hr. 93% of the recovered radioactivity is retained in the tissue when a 21 hr treatment with digitonin is followed by partial dehydration. Results are ± standard errors except for 40 hr exposure to digitonin when only two measurements were made. Frozen section radioautograms are assumed to contain 100% of the radioactive cholesterol.

(HD) units. One HD unit is the distance from a line source within which half of the total developed grains falls (32); it was taken as 0.5 μ for the light microscope radioautograms (13). In this form the grain distribution could be compared with the theoretical distribution expected if all the radioactivity in the alveolar lumen came from the alveolar wall. The theoretical distribution was obtained from the data of Hedley-Whyte et al. and Salpeter et al. (13, 32).[2]

[2] The "edge" compartment was assumed to be a solid straight band of width 1.33 HD units. The data were normalized to unity over the whole edge compartment, which meant that the theoretical curves for solid band sources could not be used exactly because they referred to data normalized over a line at the edge of the source (32). The modified values of Fig. 12 of reference 32, used by Hedley-Whyte et al., were therefore used, as in Table III of the study by Hedley-Whyte et al (13). These values gave an approximate grain distribution because they referred to a normalized compartment of approximately 4 HD units rather than 1.33 HD units. However, Fig. 12 of Salpeter's study (32) shows that grain distribution around sources of half widths 0.5 – 4.0 HD units is essentially identical.

For analysis of electron microscope radioautograms, micrographs were obtained of a large number of sections by photographing preassigned areas on the grid. Electron micrographs were superimposed with a lattice of points spaced 2 cm apart. Grains and points were counted over red cells and lipid droplets of septal cells. The lamellar bodies of the type II alveolar epithelial cells were not included in the lipid droplet count. Grains and points over the whole alveolar wall, the type II alveolar epithelial cell and the alveolar macrophage, were also tabulated. Grain densities were compared after conversion of the whole alveolar wall value to unity and multiplication of the other densities by the same factor.

RESULTS

The distribution of radioactivity was expressed in terms of relative grain density (grains per points). Despite the variations, from 16 to 100%, in the amount of radioactivity remaining in the tissue, our methods of analysis permitted an examination of the distribution of the administered radioactive cholesterol in the lung and a comparison of the distribution of radioactivity after the four methods
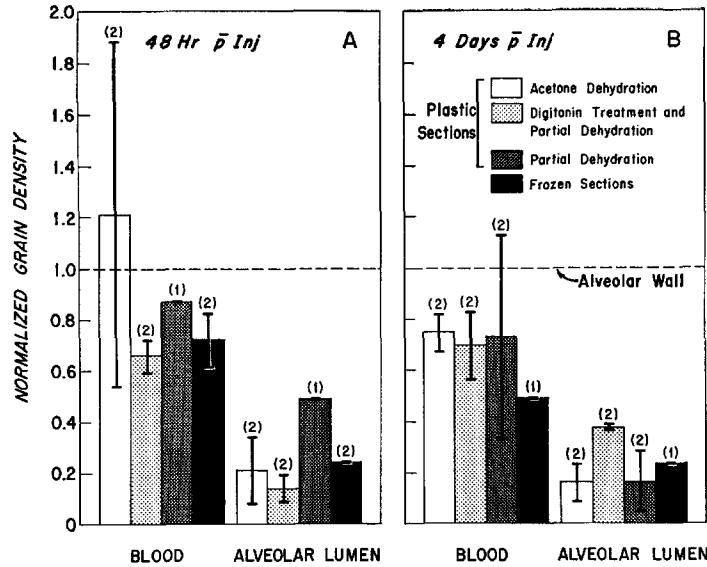
FIGURE 2 (A) Distribution of cholesterol-1,2-^{3}H 48 hr after injection in light microscope radioautograms of Epon sections of lungs from two mice and in frozen sections of lungs from two mice. All mice were 8 days old at time of injection. (B) Distribution of cholesterol-1,2-^{3}H in radioautograms from Epon sections of lungs of two mice 4 days after injection and frozen sections of lungs of a mouse 5 days after injection. Grain densities over blood vessel lumina (more than 1 μ from endothelium), alveolar wall, and alveolar lumen (more than 1 μ away from alveolar wall) are compared after normalization of the grain densities to unity over the alveolar wall. Note the similarity in distribution between all four processing methods. Variation from mouse to mouse exceeds variation between different processing methods. Values are expressed as mean with range and number of animals indicated. The standard deviation of the alveolar wall compartment ranged from ± 0.07 to ± 0.25. The grain density in the blood vessel which can be accounted for by spread of radioactivity from alveolar wall is less than 0.13 (13, 32).

of preparing the lung sections for radioautography (Fig. 1).

In general, the grain density distribution in light microscope radioautograms of cholesterol-1,2-^{3}H in mouse lung at any one time interval after injection was similar, except for the alveolar lumen, after all four methods of processing (Figs. 2–5). The differences between the grain density distributions fell mostly within the range of values found within each method (Figs. 2 and 3). The greater the number of mice sampled, the closer was the agreement of mean grain density distributions between methods (Fig. 3). Despite the wide range of values found over blood vessel lumina more than 1 μ away from the endothelium, the information obtained from each method was similar: that the radioactive cholesterol was present in blood vessels to a greater extent than could be accounted for by spread from alveolar walls (Fig. 2). The density of radioactivity in the alveolar lumen after tracheal infusion fixation showed a wide variation, but this density was greater

than could be accounted for by the sole source being in the alveolar wall, with the exception of the nondigitonin preparations at 4 days after injection (Fig. 4). The analysis of the frozen section radioautograms supported the conclusion that cholesterol-1,2-^{3}H was present in the alveolar lumen. The differences between the relative alveolar wall grain densities with and without digitonin treatment were not significant, and both values were in the same range as those in the frozen section radioautograms (Fig. 4).

Grain density distributions over the alveolar lumen in frozen sections from 2 and 5 days after injection were significantly higher than the theoretical values for density distributions around a band source (32), in this case the alveolar edge, indicating a radioactive source within the alveolar lumen (Fig. 4). No evidence for a radioactive source at the edge of the alveolar lumen was found at 15 days after injection in frozen section radioautograms. The alveolar lumen grain density, more than 3.5 HD from the alveolar wall, was
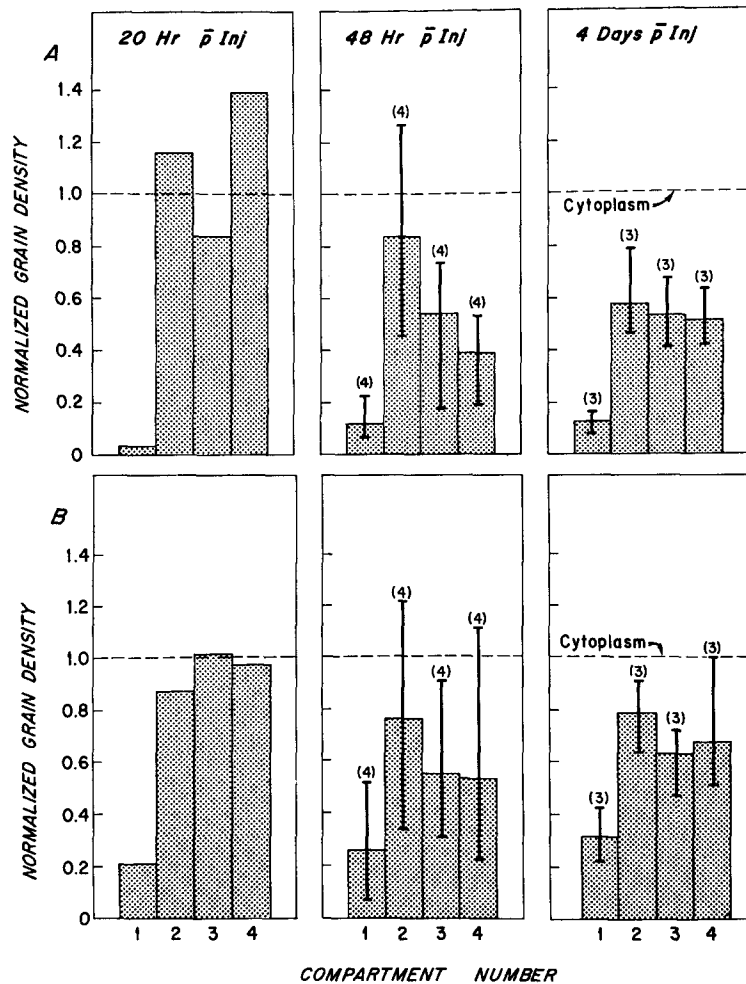
FIGURE 3 Distribution of cholesterol-1,2-^{3}H in mouse lung in light microscope radioautograms after (A) acetone dehydration (16% [20 hr] or 51% [48 hr and 4 days] retention of radioactivity in the tissue) and (B) digitonin treatment followed by a partial dehydration (93% retention of radioactivity in the tissue). The compartments represent: 1, alveolar lumen; 2, epithelial edge; 3, capillary lumen; 4, nucleus; and 5, cytoplasm (the normalized compartment) (see Methods). The bars represent the mean normalized grain densities with range and number of mice indicated. There is agreement between the two processing methods with the exception of the cytoplasm to alveolar lumen grain density ratio which is consistently less in the digitonin preparations at all three sacrifice times. The standard deviation of the cytoplasmic compartment ranged from ±0.05 to ±0.13.

consistently higher than the theoretical value at both 2 and 5 days after injection. Distribution of grains over the frozen sections was the same whether the sections were fixed with Wohlman's fixative or formalin vapor.

The presence of radioactive cholesterol at the edge of the alveolar lumensuggested that this cholesterol was present in pulmonary surfactant within 2 days of injection into 8-day old mice. The surfactant, with its cholesterol, was apparently

displaced by tracheal infusion fixation (Fig. 4). Lipid vacuoles in alveolar septal cells were found to be highly labeled 2 and 4 days after injection. The radioactive cholesterol was found particularly at the periphery of the vacuoles in both the digitonin- and nondigitonin-treated lungs (Figs. 6 and 7, Table I). The lamellar bodies in the type II alveolar epithelial cells were not more labeled than the surrounding cytoplasm (Fig. 6).

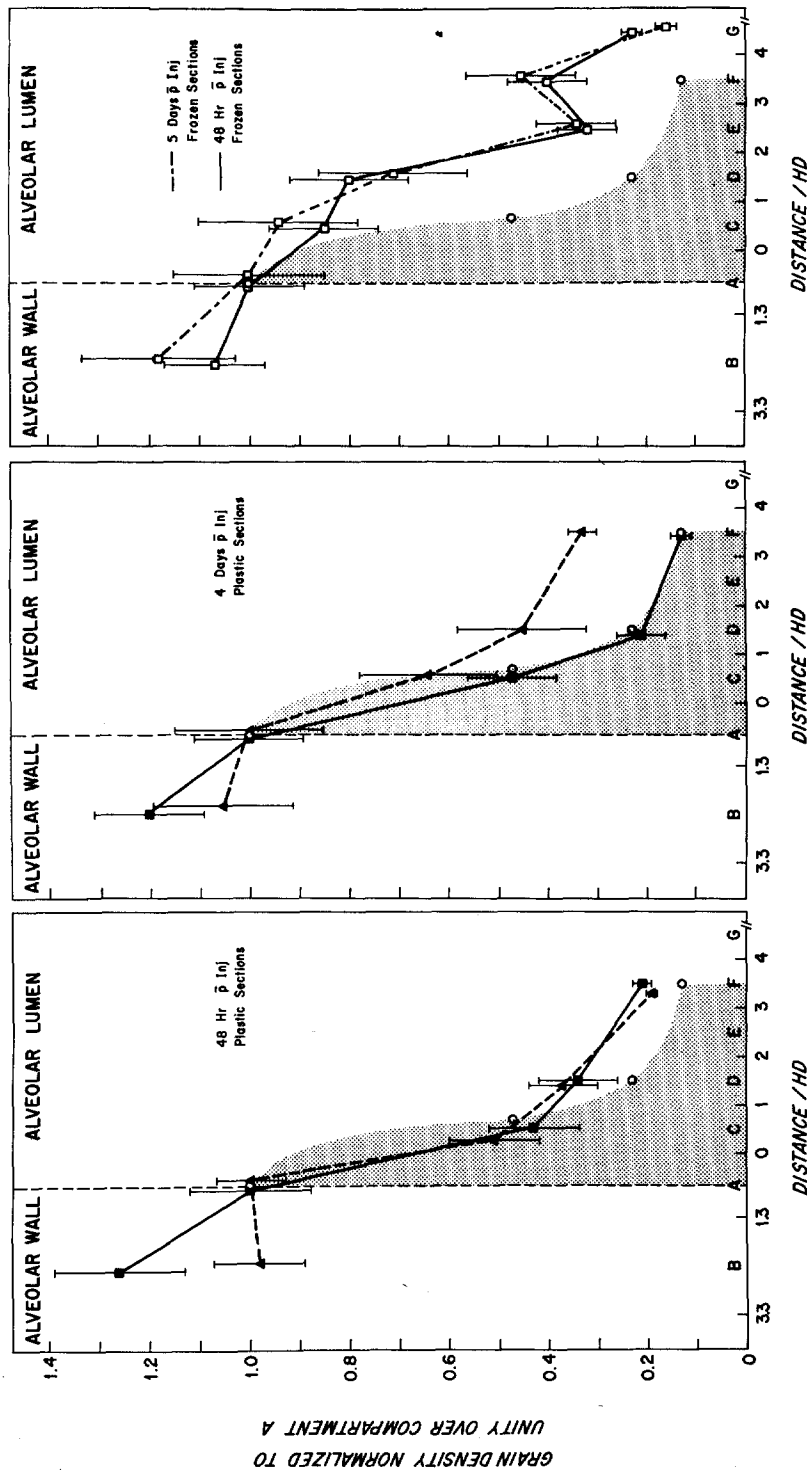Radioactive cholesterol was taken up into

FIGURE 4 Distribution of cholesterol-1,2-³H around the edge of the alveolar lumen in light microscope radioautograms from mice injected aged 8 days. Closed squares represent values from radioautograms processed by acetone dehydration with a 24 hr postfixation in Dalton's chrome osmium (51% retention of radioactivity in the tissue). Closed triangles represent values from radioautograms processed by a digitonin treatment followed by partial dehydration (93% retention of radioactivity in the tissue). Open squares represent values from frozen section radioautograms (100% retention of radioactivity in the tissue). Open circles represent theoretical values calculated on the assumption that all the radioactivity is located in the alveolar wall (32). Grain densities in compartments C and D in both types of plastic section radioautograms agree well with the theoretical values, suggesting that there is no source of radioactivity just outside the alveolar wall. This is contrary to the situation in the frozen section preparations where grain densities in compartments C and D are significantly higher than the theoretical values. Grain densities over the alveolar lumen (compartment G) exceed the theoretical value in all but one case. Relative grain densities over the alveolar wall (compartment B) are the same for all preparations, but those over the alveolar lumen (compartment G) are not. This suggests that the loss of radioactivity from the alveolar lumen may vary according to the processing technique used. For explanation of compartments A-G, see analysis III, Methods. The edge of the alveolar wall is approximately the midpoint of compartment A. One-half distance (HD) is the distance from a line source within which half of the total developed grains falls (32); for this study it has been taken as 0.5 μ. Each point represents data totaled from two mice and expressed ± standard deviation.
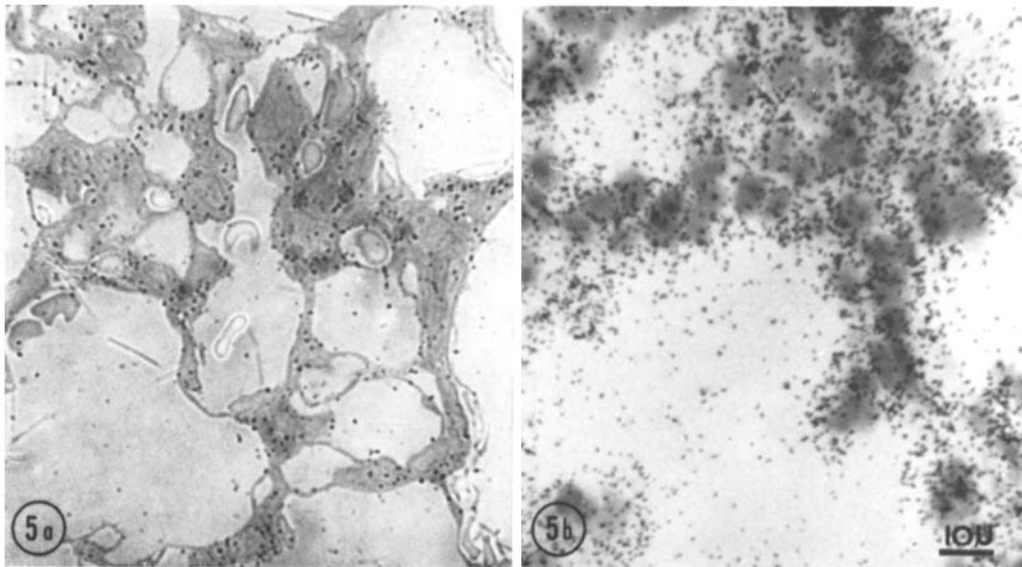
FIGURE 5   Light microscope radioautograms of lungs of 10-day old mice injected aged 8 days with choles-terol-1,2-^{3}H. Fig. 5 *a* is a 1 μ Epon section radioautogram prepared from acetone-dehydrated tissue postfixed 24 hr in Dalton's chrome osmium. Fig. 5 *b* is a 2 μ frozen section radioautogram prepared by the Appleton technique. X 700.

mouse lungs very rapidly in the first 24 hr after a single intraperitoneal injection and then more gradually over the next 3 days. By 5 wk after injection, radioactive cholesterol was still detectable in the lung. The amount of radioactive cholesterol present in the lung at 2 days after injection into an 8 day old mouse was sufficient to obtain an electron microscopic radioautograph with 4 months of exposure (Figs. 7, 8).

Grain density distribution in electron microscope radioautograms was similar whether the tissue was processed with or without digitonin (Table I). Grain densities over the red blood cells ranged from 0.26 to 0.51 of the value over the whole alveolar wall for the acetone-dehydrated sections and from 0.41 to 0.62 for the sections treated with digitonin. Both tissue processing methods gave rise to high relative density values over the lipid droplets, 1.90–2.46 without digitonin and 1.30–2.65 with digitonin. Both methods gave grain densities over the type II alveolar epithelial cells that were the same or half the value over the whole alveolar wall. Both with and without digitonin some alveolar macrophages were highly labeled; others were not labeled at all.

Grain density over nonradioactive sections was randomly distributed and not significantly greater than background grain density for all processing techniques employed, indicating that positive chemography had not occurred. Sections that had been partially dehydrated without prior precipitation of cholesterol by digitonin gave grain densities over areas of plastic on radioactive sections that were less than twice background values.

If sections that had been partially dehydrated were coated with emulsion without prior coating of the sections with carbon, latent image fading occurred over tissue areas. Interspersion of a carbon layer between section and emulsion caused a striking increase in the alveolar wall to alveolar lumen grain density ratio in partially dehydrated sections (Fig. 9). The grain density over the sections processed by the other methods increased linearly with increases in exposure times.

The fate of the lung radioactivity during fixation, dehydration, and infiltration for sectioning is shown in Fig. 1. Loss in the pooled fixatives and Sabatini's washing solution was found to be consistently less than 1% (Fig. 1). After acetone dehydration and propylene oxide infiltration, only 15.9 ± 3.7% (SE) of the total radioactivity recovered from the tissue and all the processing fluids remained in the tissue (Fig. 1). 78 ± 3.8%

TABLE I

Distribution of Cholesterol-1,2-³H in Mouse Lung Electron Microscope Radioautograms

Grain density (per unit area) normalized to unity over alveolar wall

Age at inj / Time p̄ inj	Complete acetone dehydration					Digitonin treatment				
	Alveolar wall	Red blood cell	Lipid droplets	Alveolar macrophage	Type II AEC*	Alveolar wall	Red blood cell	Lipid droplets	Alveolar macrophage	Type II AEC
8 days / 48 hr	1.00 ± 0.04‡	0.51 ± 0.07	1.92 ± 0.09	0.94 ± 0.09	1.18 ± 0.16	1.00 ± 0.05	0.51 ± 0.08	2.65 ± 0.40	0.64 ± 0.15	0.99 ± 0.12
8 days / 48 hr	1.00 ± 0.06	0.49 ± 0.11	1.90 ± 0.30	—	—	1.00 ± 0.05	0.62 ± 0.09	1.97 ± 0.30	—	—
8 days / 48 hr	1.00 ± 0.05	0.32 ± 0.06	2.39 ± 0.50	—	1.05 ± 0.09	1.00 ± 0.05	0.41 ± 0.17	1.30 ± 0.30	0.97 ± 0.20	0.54 ± 0.07
8 days / 4 days	1.00 ± 0.06	0.40 ± 0.08	2.34 ± 0.45	1.94 ± 0.28	0.57 ± 0.08	1.00 ± 0.09	0.54 ± 0.11	2.40 ± 0.60	1.38 ± 0.30	0.95 ± 0.11
8 days / 4 days	1.00 ± 0.05	0.33 ± 0.06	2.46 ± 0.40	0.97 ± 0.16	0.92 ± 0.08	—	—	—	—	—
8 days / 4 days	1.00 ± 0.08	0.26 ± 0.06	2.15 ± 0.45	—	0.91 ± 0.20	1.00 ± 0.07	0.53 ± 0.12	1.41 ± 0.34	0.92 ± 0.18	0.50 ± 0.10

* AEC, alveolar epithelial cell.

‡ Values are ±standard deviations calculated from the formula $G/P\sqrt{(1/\sqrt{G})^2 + (1/\sqrt{P})^2}$ (multiplied by the same factor used to convert grain density over alveolar wall to unity).
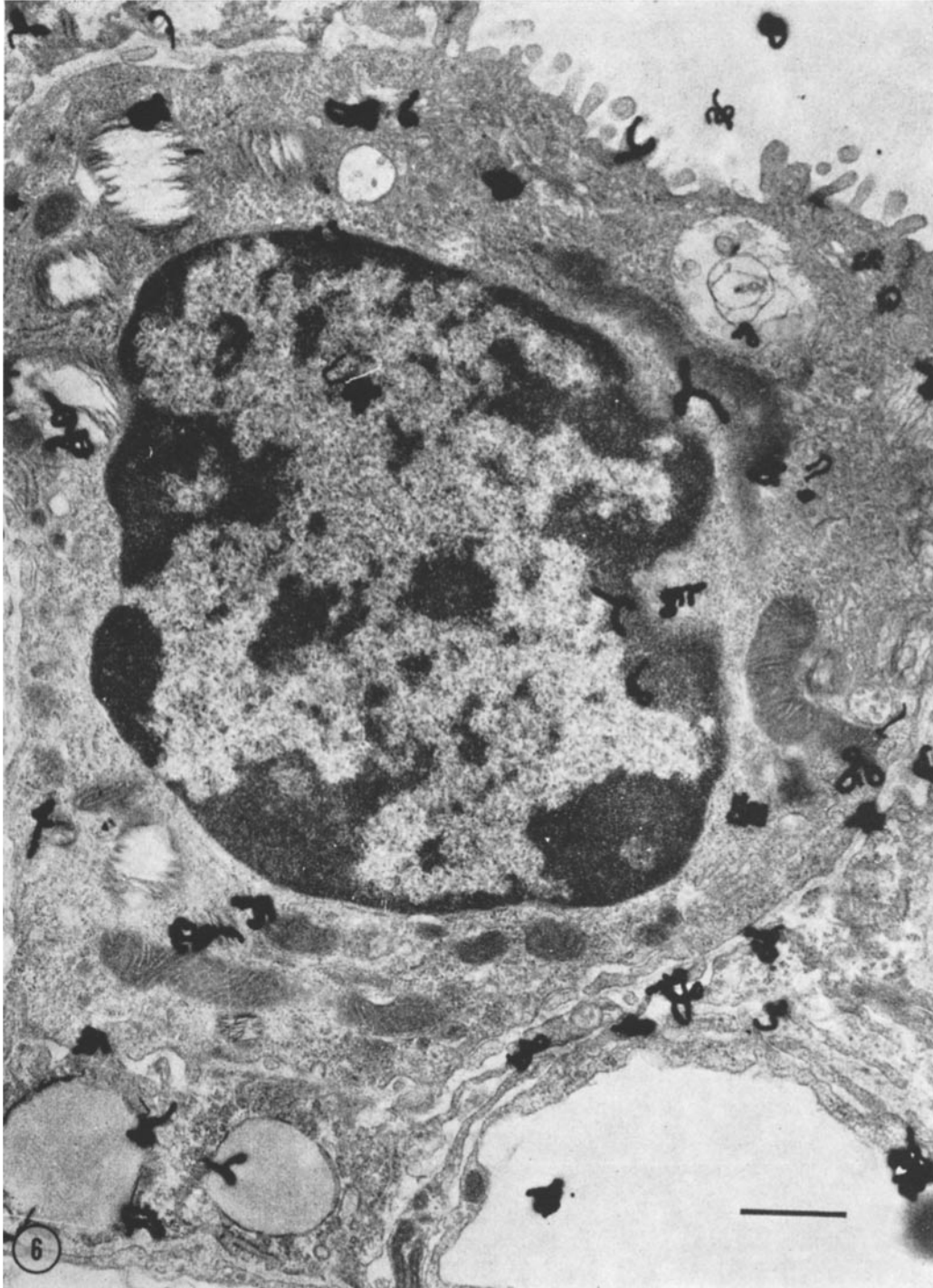
FIGURE 6 Electron microscope radioautogram of cholesterol-1,2-³H in the type II alveolar epithelial cell of the lung of a 12 day old mouse sacrificed 4 days after injection. The tissue was dehydrated with acetone after 24 hr postfixation in Dalton's chrome osmium. Note that the silver grains are located randomly throughout the cell, and are not preferentially attached to the lamellar bodies. 6 months of exposure to Ilford L4 emulsion. Development was with Microdol-X. Scale bar, 1 μ. ×15,300.
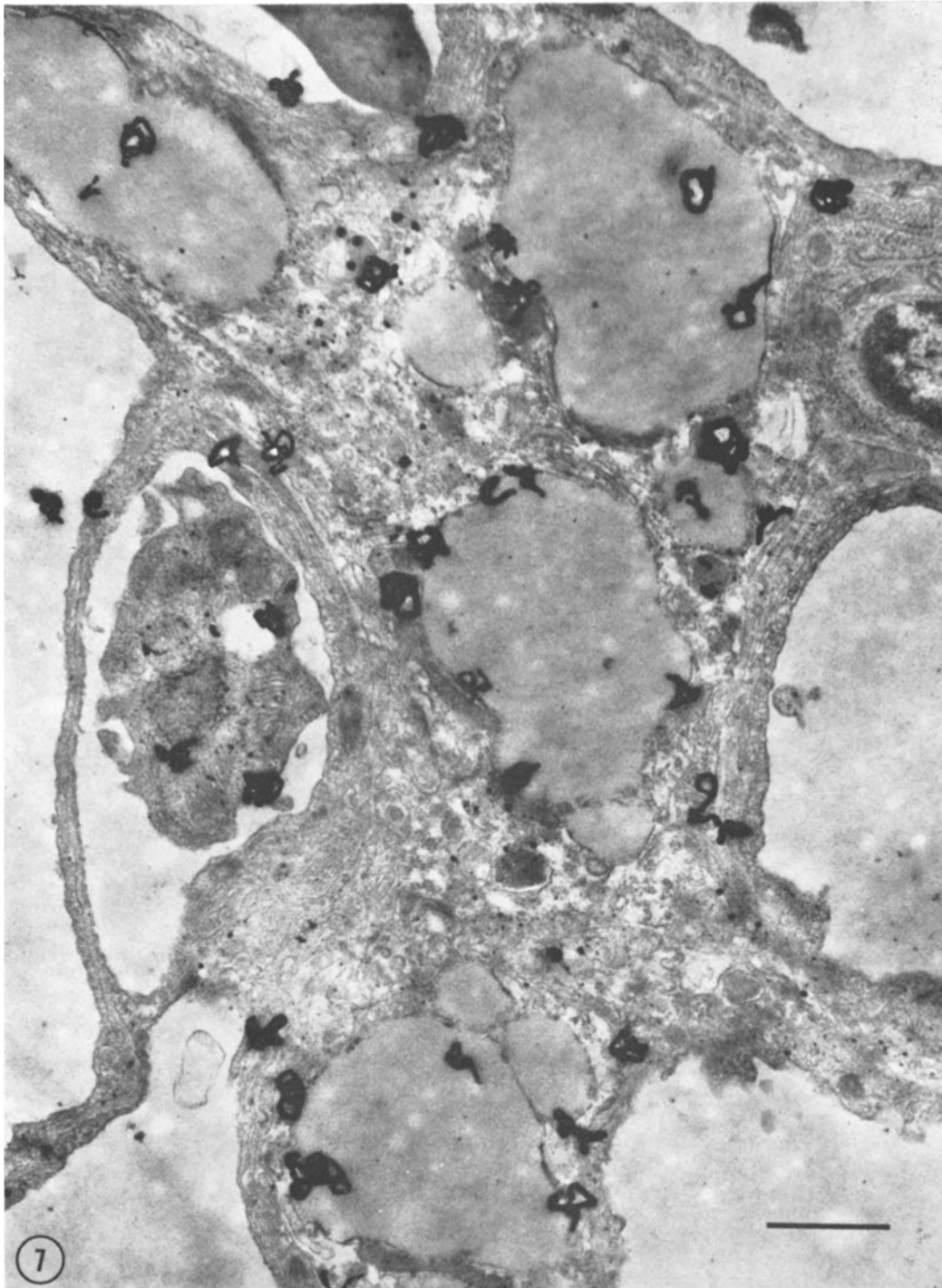
FIGURE 7 Electron microscope radioautogram of cholesterol-1,2-³H in the lung of a 10 day old mouse sacrificed 2 days after injection. The tissue was processed with a 21 hr digitonin treatment followed by a partial dehydration. The grains are assoicated with the lipid vacuoles and concentrated towards the periphery. 4 months of exposure to Ilford L4 emulsion, Microdol-X developed. Scale bar, 1 μ. × 17,600.
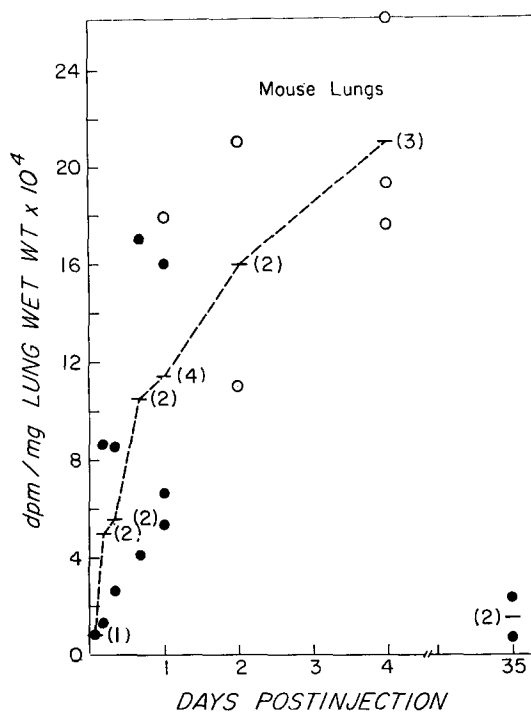
FIGURE 8 Uptake of cholesterol-1,2-³H into mouse lungs after intraperitoneal injection. Each closed circle represents values from one mouse, initial dose 160–180 μCi. Each open circle represents values from one mouse, initial dose ~250 μCi. The interrupted line represents the means of the values from the numbers of mice indicated in parentheses.

(SE) of the recovered radioactivity was found in the acetone and propylene oxide solutions, and 4.9 ± 1.0% in the propylene oxide–Epon infiltrating solution. Radioactivity retained in the tissue was increased to 51.4 ± 4.6% when postfixation with Dalton's chrome osmium was extended from 1 to 24 hr.

Partial dehydration (35) resulted in 63.1 ± 4.5% retention of the radioactivity in the lung tissue. The amount of radioactivity lost in the Epon infiltrating mixture was almost as much as that in the dehydrating solutions: 15.7 ± 3.7% versus 20.1 ± 4.2%. When tissues were dehydrated in increasing concentrations of Durcupan in water and then infiltrated and embedded in Durcupan, only 29 ± 6.5% of the recovered radioactivity remained in the tissue after processing (Fig. 1).

Digitonin treatment followed by partial dehydration resulted in a retention of radioactivity in the tissue of 93.4 ± 1.13% (Fig. 1). Digitonin

treatment followed by dehydration in graded acetones and propylene oxide preserved only 43.5 ± 2.9% of the recovered radioactivity in the tissue (Fig. 1); the percentage was increased to 56% with a 40 hr treatment in digitonin. The average recovery of radioactivity was 76%.

Preservation of tissue architecture was best with acetone dehydration and propylene oxide infiltration (Fig. 6), but partial dehydration after digitonin treatment resulted in adequate preservation and the tissue contained the most radioactive cholesterol for radioautographic studies (Fig. 7).

DISCUSSION

Radioautograms of cholesterol-1,2-³H in lung parenchyma showed a grain density distribution which was essentially the same whether 16, 63, 93, or 100% of the radioactivity was retained in the lung. Cholesterol was therefore lost randomly from the parenchyma and was not redistributed within the tissue during histological processing. Severe latent image fading, which occurred in sections that had been partially dehydrated, was prevented by the use of a carbon layer between section and emulsion. 93% of the labeled cholesterol was retained in the tissue by a digitonin treatment followed by a partial dehydration in alcohol and Epon at 0°C.

The finding of a radioactive source in the alveolar lumen adjacent to the alveolar edge suggested the presence of radioactive cholesterol in pulmonary surfactant, an observation which we have confirmed by chemical analysis of lung washings in rats after intraperitoneal administration of cholesterol-1,2-³H (9). Lipid vacuoles in alveolar septal cells were more radioactive than the surrounding cytoplasm, indicating an increased concentration of cholesterol in the lipid vacuoles. The lamellar bodies showed no particular concentration of radioactivity at the time intervals examined, so that we have not demonstrated their role in the metabolism of surfactant. It seemed unlikely from these experiments that they were the source of the radioactive cholesterol in the surfactant layer. The finding of scattered, highly labeled alveolar macrophages, sufficiently hot to be identified without grain density analysis, was intriguing. The data obtained in these experiments did not permit any conclusion as to whether these cells are the source of surfactant material or whether they are removing it (9). Similar experiments conducted with different time intervals might help to elucidate this problem.
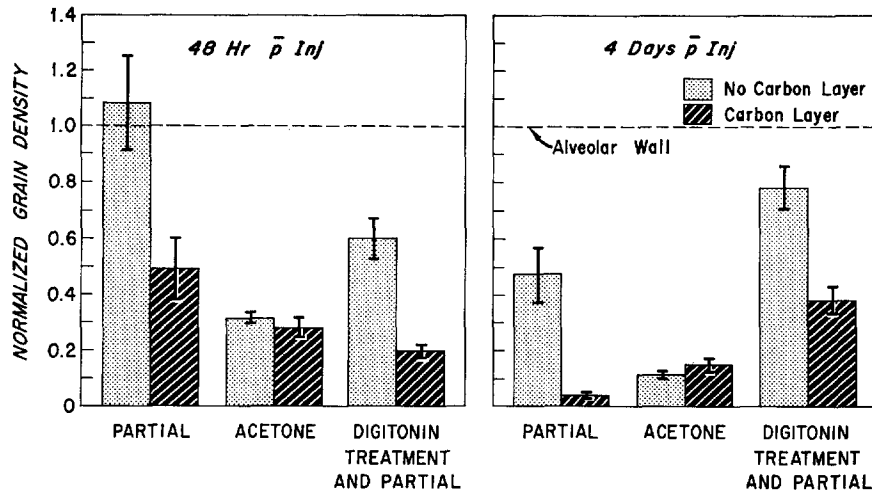
DEHYDRATION

Figure 9   Density of cholesterol-1,2-^{3}H in the alveolar lumen of mouse lungs in light microscope Epon-section radioautograms after different methods of dehydration and infiltration. Grain densities are normalized to unity over the alveolar wall. Note that the interspersion of a carbon layer between section and emulsion causes a striking increase in the alveolar wall to alveolar lumen grain density ratio in tissues that have been partially dehydrated prior to embedding. Discrepancies in grain density distributons occurred because of latent image fading over tissue areas in partially dehydrated tissue. Values are ± standard deviation; the average standard deviation of the alveolar wall compartment is ±0.13.

Major variations in grain density distributions occurred over the blood vessel and alveolar lumina. Varying grain density distributions over blood vessels were probably related to the degree of inflation of the lung and the amount of blood which escaped from the tissue prior to fixation. Grain density over the blood vessel lumen in the frozen section radioautograms was always highest in the first lobe excised and frozen.

Variations in relative grain densities over the alveolar lumen suggested that the method of fixation was of primary importance in the study of substances within the alveolus. Intratracheal infusion of glutaraldehyde probably infiltrated alveoli unevenly and led to uneven washing out of the surfactant which contains cholesterol (9, 16, 23). Cell damage secondary to freezing in the Appleton technique might have allowed diffusion of the intracellular cholesterol into the alveolar lumen (21, 34). However, this possibility probably was not important since it has been shown chemically that radioactive cholesterol was present in lung washings after intraperitoneal administration of radioactive cholesterol to rats at similar time intervals (9). Physical interaction between tissue edge and emulsion, the edge effect, might also

have led to an erroneously high grain density adjacent to the alveolar wall in frozen sections but not in Epon sections (4). Frozen section radioautograms from animals sacrificed 15 days after injection did not reveal a radioactive source adjacent to the alveolar wall, hence diffusion and edge effect were not significant. Different dehydration and infiltration methods had less effect on grain density distribution than variation in method of fixation (Figs. 2, 3, and 4). The close agreement in relative grain density distributions between the digitonin- and nondigitonin-treated preparations indicated that migration of cholesterol within the tissue during dehydration and infiltration did not occur.

The frozen section radioautographic technique was useful for studying radioactive sources in the alveolar lumen, but cellular resolution was poor. Frozen section radioautograms prepared by the Appleton technique (1) gave reproducible results; the variation in relative grain density distributions between mice sacrificed at the same time seldom exceeded 20%. The frozen sections adhered well to the emulsion-coated slides. The resolution of frozen section radioautograms with tritium has not been experimentally determined; it was assumed to be 0.5 μ. For sections less than 1 μ thick, grain

density was halved at a distance of 0.5 μ from a tritium source (2, 4). At 15 days after injection fall-off in grain density from the alveolar wall was in close agreement with the theoretical values which assumed a resolution of 0.5 μ (32). The analyses of the distribution of grain density around the alveolar wall were limited in accuracy because of the hazy appearance of the tissue edge. The frozen section radioautograms were intended as controls for the Epon-embedded radioautograms, on the basis of the assumption that such a technique would not allow any migration or loss of cholesterol from lung tissue before or during exposure of the radioautograms.

The use of cholesterol-1,2-³H and lung for radio-autography did not give rise to any positive chemography. However, latent image fading over tissue areas was a serious problem in sections that had been partially dehydrated and coated with emulsion without an intervening carbon layer. It was probably related to the presence of moisture in the tissue (27). A severe latent image fading effect has been observed in the skin and sub-mandibular gland in frozen sections stored in contact with emulsion at −30°C (18, 28). We did not observe bleaching on slides that had been exposed to light prior to storage at −85°C.

Severe loss of cholesterol from liver (97%), nerve (99%), and adrenal cortex (99%) has occurred during histological processing of tissue for electron microscopy (14, 24 and 33), whereas only 14% of cholesterol was leached from red blood cell stroma by similar treatment (22). These observations demonstrated that loss of cholesterol varies greatly according to the tissue examined, a situation which is probably true for other compounds as well. With a complete acetone dehydration, we found that only 16% of the radioactive cholesterol was retained in the lung. This retention was increased to 51% when postfixation with Dalton's chrome osmium was increased from 1 to 24 hr. Frühling et al. made similar observations on the adrenal cortex and found that precipitation of cholesterol digitonide was only successful in retaining cholesterol if the tissue was partially dehydrated with alcohol and Epon at 4°C (10, 15). We obtained the same results with cholesterol in lung tissue. The partial dehydration technique without prior precipitation of cholesterol by digitonin in the lung gave rise to extensive loss of cholesterol, which has also been found with liver (36). The use of Durcupan failed to retain choles-

terol in the lung, as well as in liver and nervous tissues (14).

In soluble compound radioautography, extent of retention and migration of the radioactive compound may or may not be related. These comparative analyses of grain density distributions in radioautograms of lung after administration of cholesterol-1,2-³H led to the conclusion that radio-autograms from Epon-embedded tissue were valid for the intracellular localization of cholesterol in the lung. We concluded that cholesterol was lost randomly from the tissue during dehydration and infiltration rather than being redistributed. The radioautograms from air-inflated lungs demonstrated that the methods of inflation and fixation were of primary importance in determining grain distribution within the alveolar lumen and that conclusions about intra-alveolar distribution of cholesterol after glutaraldehyde infusion fixation are essentially impossible. Tissues labeled with potentially soluble compounds should be processed for radioautography by at least two different techniques chosen to minimize loss of radioactivity and prevent migration. All steps should be monitored with scintillation counting, chemical analysis, and comparative analysis of radioautographs.

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