RADIOAUTOGRAPHIC DEMONSTRATION OF 5-HYDROXYTRYPTAMINE-3H UPTAKE BY PULMONARY ENDOTHELIAL CELLS

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ABSTRACT

The lung is able to rapidly remove 5-hydroxytryptamine (5-HT) from the circulation by a Na+-dependent transport mechanism. In order to identify the sites of uptake, radioautographic studies were done on rat lungs which had been isolated and perfused with 5-HT-3H and 0.5 mM iproniazid, a monoamine oxidase inhibitor. In control experiments 10-4 M imipramine was added to the perfusate to inhibit the membrane transport of 5-HT. At the light microscope level, silver grains were seen concentrated near capillaries and in the endothelium of large vessels. From electron microscope radioautographs a semiquantitative grain count was made and 90% of the silver grains were observed over capillary endothelial cells. The grains were found over the nucleus and cytoplasm of the cell and showed no preferential association with any particular cytoplasmic inclusion bodies, organelles, or vesicles. Other cell types were unlabeled except for a few mast cells, certain vascular smooth muscle cells, and one nerve ending. This radioautographic demonstration of the cell type responsible for the rapid removal of 5-HT from the lung circulation clearly establishes the existence of a new metabolic role for pulmonary endothelial cells.

INTRODUCTION

The lung serves several important nonrespiratory functions. One of these is its role in the metabolism of various circulating substances such as peptides, lipids, nucleotides, and biogenic amines (8, 27). The removal of 5-hydroxytryptamine (5-HT, serotonin) from the blood was first demonstrated convincingly in 1953 (5). The importance of this phenomenon has been confirmed in more recent studies in which up to 95% of the 5-HT infused intravenously into dogs in vivo or into the pulmonary circulation of rat lungs in vitro was inactivated in one passage through the lungs (1, 23). When 5-HT-1C is perfused through the pulmonary circulation of isolated rat lungs it is rapidly removed from the circulation and becomes concentrated in the tissue. One of us has currently shown that this uptake is a saturable process and that its rate-limiting step is the transport of 5-HT into the cell (12). Moreover, it was shown that 5-HT transport is inhibited not only by drugs such as imipramine, chlorpromazine, and cocaine (known to inhibit the membrane transport of biogenic amines), but also by decreasing or removing extracellular Na+ and by increasing extracellular K+. From these observations it was concluded that a Na+-dependent, carrier-mediated transport system for 5-HT was operating in the lung (12). Similar conclusions had been reached in studies on the uptake of norepinephrine (NE) by rat heart slices (2), the transport and accumulation of NE and 5-HT by brain synaptosomes (25), and the uptake of 5-HT by platelets (21).
The pharmacological study on the uptake and metabolism of 5-HT-3H in isolated perfused rat lungs (12) suggested that neither platelets, nor mast cells, nor adrenergic nerve endings were likely candidates for the site of uptake of circulating 5-HT. Pulmonary endothelial cells were thought to be responsible for this rapidly occurring phenomenon because they provide a vast surface area in contact with the blood or perfusion medium. To verify this hypothesis, a light and electron microscope radioautographic study of lungs perfused with 5-HT-3H was undertaken.

MATERIALS AND METHODS
Male, Sprague-Dawley rats, weighing 250-350 g, were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg, body weight). A tracheotomy was performed and a cannula inserted into the trachea for mechanical ventilation of the lungs with a mixture of 5% CO2-95% O2. The thorax was opened and the lungs were perfused at a rate of 10 ml/min, isolated, and placed into a moist closed chamber (by a procedure previously described (12)). The perfusion medium consisted of Krebs-Ringer bicarbonate buffer containing 5 mM glucose, 4.5% bovine serum albumin, and 0.5 mM iproniazid,1 the iproniazid serves to prevent the oxidation of 5-HT to 5-hydroxyindole acetic acid (12). The perfusion medium was equilibrated with 95% O2-5% CO2 so that the pH was 7.4. After perfusing the lungs for 10 min with the standard medium (equilibration period), 1.1-12 mM 5-HT-3H creatinine sulfate (specific activity, 2.2 and 12 Ci/m mole, Amersham-Searle Corp., Arlington Heights, Ill.) was infused through the pulmonary circulation for 3 min in a known volume of the same medium. The amount of radioactivity infused varied from 400 mCi to 800 mCi A representative sample of the inflow and the effluent were collected during each 3 min infusion period, and the difference in radioactivity between these two samples was used as an estimate of the amount of 5-HT-3H taken up by the lung.

Two control experiments were done to check the specificity of the uptake of radioactivity by the lung. They involved the addition of imipramine1 (at a final concentration of 10-4 M) to the perfusion medium to inhibit the uptake of 5-HT-3H by the lung (12).

The purity of the tritium-labeled 5-HT was checked by extracting a sample of the inflow with 2 vol of methanol-sec-tone (1:1), and centrifuging it at 4°C for 20 min at 10,000 g. The supernatant fluid was then analyzed by thin layer chromatography on glass plates coated with Silica-gel F 254, using isopropanol-methyl acetate-ammoniac (35:45:20) as the solvent system (22).

For measuring radioactivity, samples of inflow, effluent, and lung tissue were placed into scintillation vials and dissolved in NCS solubilizer (Amersham-Searle Corp.). 10 ml of a scintillation mixture consisting of 7 g of 2,5-diphenyloxazole (PPO) and 0.1 g of p-bis-(p-methyl styryl)-benzene (bis-MSB) in 1 liter toluene was then added and the radioactivity was measured in a Packard liquid scintillation spectrometer. An internal standard was used to monitor the efficiency of counting.

Electron Microscopy and Radioautography
After the infusion of 5-HT-3H, the lungs were fixed by perfusing the pulmonary circulation with 2.9% glutaraldehyde (and, in one experiment, 3% paraformaldehyde) in 0.133 M sodium phosphate buffer, pH 7.4, or 0.133 M sodium cacodylate buffer, pH 7.4. The fixative was perfused for 10 min, and then representative areas from each lobe of the lung were taken and cut into small pieces for an additional 2 hr fixation at room temperature. The tissue was transferred to cold buffer for 18 hr, and then postfixed at 4°C in 2% OsO4 (prepared in the same buffer used for primary fixation). After dehydration in a graded series of ethanol, the pieces were embedded in Epon (13). In three experiments, larger pieces of fixed lung were excised, dehydrated, and embedded in paraffin.

For radioautography at the light microscope level, 1 µ thick Epon sections were cut (6-µ thick sections were cut from paraffin-embedded material) and placed on gelatin-alum-coated slides. The slides were dipped into melted Ilford L4 emulsion (Ilford Ltd, Ilford, Essex, England) diluted 1:1 with water, dried, and stored in light-tight boxes containing Drierite. After exposure for 1-2 wk at 4°C, the sections were developed in Dektol (Eastman Kodak Co., Rochester, N Y), cleared in Kodak Hypo, washed in water, and stained with 1% toluidine blue “O” buffered to pH 7.2.

For electron microscope radioautography, light gold sections were cut on a Sorvall MT-2 ultramicrotome, and placed on carbon-coated copper grids. The loop method of Caro and van Tubergen (3) was used to coat the thin sections with Ilford L4 emulsion. After exposure for 3-4 months at 4°C, the sections were developed for 5 min in Kodak Microdol-X, cleared in Kodak Rapid-fixer, washed, and stained with 1% uranyl acetate in 30% ethanol (3) for examination in a Philips EM 300 electron microscope.

1 Imipramine phosphate was donated by Hoffmann-La Roche, Inc., Nutley, N. J., and imipramine hydrochloride was a gift of Geigy Pharmaceuticals, Ardsley, N. Y.
FIGURES 1 and 2  Light micrographs of 1 μ thick Epon-embedded sections of rat lung.

FIGURE 1  This lung has been perfused with 5-HT-3H. Silver grains (arrow), representing the isotope, are seen concentrated near capillaries. X 1000.

FIGURE 2  Imipramine was present during perfusion of this lung with 5-HT-3H. Silver grains are rare since imipramine blocks 5-HT-3H uptake. X 1060.
RESULTS
The uptake of 5-HT-3H by eight lungs perfused with the isotope in the absence of imipramine ranged from 11 to 40% when expressed as a percentage of the total amount of 5-HT-3H infused. During perfusion fixation with glutaraldehyde, 14.3 ± 3.3% of the radioactivity taken up by the lung was lost (mean of four experiments ± se). The loss of 5-HT-3H from fixed pieces of lung during subsequent steps in processing was 7.5 ± 0.3% during the additional 2 hr glutaraldehyde fixation, 2.1 ± 0.15% during 18 hr storage in cold buffer; and 3.6 ± 0.25% during dehydration in a graded series of ethanol (mean of six experiments ± se). In the two control experiments with imipramine, there was no measurable uptake of 5-HT-3H.

Light Microscope Radioautography
Radioautographs of lung tissue perfused with 5-HT-3H and prepared for examination in the light microscope displayed many silver grains concentrated near capillaries (Fig. 1). Some cells were heavily labeled, whereas others remained essentially free of silver grains. A few scattered background grains were also present (Tissue fixed in paraformaldehyde showed the same distribution of silver grains as tissue fixed in glutaraldehyde).

Lungs perfused with 5-HT-3H in the presence of 10^{-4} M imipramine were essentially free of radioactivity (Fig. 2). Silver grains were rarely observed, and their numbers did not noticeably exceed that of background (compare Figs 1 and 2). In areas of the 5-HT-3H-labeled lung where larger vessels were present, the concentration of silver grains within the endothelial layer was striking (Fig. 3). The black grains literally filled the endothelial cells lining the vessels. No apparent uptake by vascular smooth muscle cells or by bronchi was detected in sections observed in the light microscope.

Electron Microscope Radioautography
The sites of 5-HT-3H labeling were conclusively identified by electron microscopy. Low magnification electron microscope radioautographs clearly showed silver grains located over endothelial cells.
(Figs. 4, 7). In unstained and lightly stained sections, the arrangement of the black silver grains dramatically outlined the luminal borders of the capillaries.

The precise location of silver grains over capillary endothelial cells is seen at higher magnifications (Figs. 5, 6). In Fig 5, portions of silver grains also lie over the basement lamina separating the alveolar epithelium from the capillary endothelium. The silver grains are sometimes so large that they may overlie portions of the alveolar epithelial cell as well as the capillary endothelial cell. Thus, in some cases either cell could theoretically be claimed as the potential source of radioactivity responsible for the grain. However, in low magnification electron micrographs (Figs 4, 7, 9) it is obvious that the grains follow the curvature of the vessels rather than the attenuated processes of the type I alveolar cells. Additional convincing evidence comes from a random...
Figures 5 and 6  Higher magnifications confirm the location of silver grains over capillary endothelial cells. Grains are seen over mitochondria (M), granular inclusions (G), the plasma membrane (P), and the nucleus (N). Fig. 5, $\times 49,000$; Fig. 6, $\times 25,000$. 

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Figure 7 A low magnification electron microscope radioautograph demonstrates silver grains concentrated over capillary endothelial cells (E), in contrast to the type II alveolar cell (AC) and another cell (*) which contain only a few grains. × 9800.

A semiquantitative grain count made from 58 survey electron micrographs taken at 4000 magnification of 1853 silver grains counted were located over capillary endothelial cells; 3% were found over alveolar epithelial cells, 33% were over other cell types, and 37% represented background counts. As indicated in the histogram (Chart 1), 10% of the capillary endothelial cells observed were unlabeled in contrast to 86% or alveolar epithelial cells. In addition, only one or
two silver grains were observed in labeled alveolar epithelial cells, whereas endothelial cells averaged seven or eight grains per cell. The chi square test reveals how significant these differences are ($x^2 = 265.44$, 1 df, $P < 0.0001$).

77% of the total number of grains counted in endothelial cells were located over the cytoplasm, and 23% were located over the nucleus. Because a morphometric analysis was not done, these percentages should not be taken to indicate the actual distribution of grains throughout the endothelial cell. The silver grains distributed over the cytoplasm of the endothelial cells had no apparent preferential association with any inclusion bodies, organelles, or vesicles. The silver grains in the nucleus were more often observed lying over heterochromatin than over euchromatin (Fig. 8).

In addition to the endothelium, other cellular components in the 5-HT-3H-perfused lung also sometimes contained a few silver grains. Type I and type II alveolar cells (Fig. 7), one of the two nerve endings observed, and platelets displayed an occasional grain. A few granular leukocytes (Fig. 8) and some mast cells (Fig. 10) were labeled, whereas red blood cells and lymphocytes were not. Smooth muscle cells near the endothelium in large vessels also contained a few silver grains.

The ultrastructural preservation of all the perfused lungs was good. Thin sections of lungs perfused with 5-HT-3H in the presence of imipramine were carefully examined in the electron microscope for possible alterations of membranes and mitochondria, in view of the known surface activity of this drug (18). No differences were seen in the mitochondria or membranes of any cell type within the imipramine-perfused lung, and the tissue looked identical to lungs perfused without the drug.

**DISCUSSION**

It has been shown that 5-HT, formed in vivo from its precursor 5-hydroxytryptophan, is not easily washed out of the lung and may be stabilized by certain hypertonic fixatives (6). In preliminary studies we found that most of the 5-HT-3H taken up by the lung was retained after glutaraldehyde perfusion fixation and dehydration. The effect of osmication could not be evaluated by liquid scintillation counting because of the quenching effect of osmium. However, radioautographs (at the light microscope level) of tissue with and without postfixation in osmium revealed no detectable difference in the amount of labeling.

It was obvious in electron microscope radioautographs that pulmonary endothelial cells were the major sites of radioactivity. These cells were intensely labeled, in striking contrast to the other cell types. The possibility that 5-HT-3H was taken up by other cell types in the lung but was retained only by the endothelial cells is unlikely, since little radioactivity was lost during perfusion fixation. Moreover, of the radioactivity remaining after dehydration and embedding, 90% was found in endothelial cells. If 5-HT-3H was solubilized or washed out during the routine dehydration procedure, other cells and structures would be expected to display some labeling, but this was not the case. We believe that glutaraldehyde reacts with, and stabilizes, 5-HT-3H within the endothelial cells where uptake has occurred. It has been shown that glutaraldehyde may bind free amino acids to...
tissue (15), and recently this fixative has been shown to react with NE to form a dense polymer that is bound in situ in tissue sections (4). Both of these reactions with glutaraldehyde appear to depend upon the presence of a primary amino group. Because 5-HT possesses a primary amino group, glutaraldehyde presumably reacts with it to form a complex that is retained within the lung tissue.

The labeling of endothelial cell nuclei is in agreement with recent studies demonstrating that 5-HT is able to bind to the bases of nucleic acids (9, 20). It remains to be determined whether or not the endothelial cell nucleus actually plays a role in the uptake (and/or metabolism) of 5-HT.

In contrast to endothelial cell nuclei, mast cell nuclei rarely contained silver grains even though their cytoplasmic granules were labeled. This suggests that when 5-HT is stored within a cell, it is not available for binding to nucleic acids.

The absence of preferential association of 5-HT with any cytoplasmic organelle suggests that 5-HT exists free in the cytoplasm of the endothelial cells. This is supported by subcellular fractionation studies of lung homogenates, where most of the 5-HT taken up by the lung is found in the soluble fraction, and by the rapid oxidation of 5-HT by monoamine oxidase which occurs under normal conditions (12). Therefore, pulmonary endothelial cells appear to be involved in

Figure 8 A leucocyte (L) is contained within the lumen of this pulmonary capillary. The nucleus (N) as well as the cytoplasm of the endothelial cell is labeled. × 14,800.
A pulmonary capillary in a 5-HT-3H-perfused lung displays a concentration of silver grains, indicating that the endothelial cells have removed the isotope from the circulation. X 21,000

Only some mast cells in the 5-HT-3H-perfused lung are labeled. This particular one contains silver grains over several of its granules. X 12,200.
the removal and inactivation of circulating 5-HT. Under physiologic conditions, this function is probably not very important since the blood contains only small amounts of 5-HT (about 1 μM), the major portion being stored in platelets. But these processes of uptake and inactivation might become important when local release of 5-HT occurs, for example during pulmonary embolism (24). Then, the pulmonary endothelial cells would be exposed to high concentrations of 5-HT and their blood-clearing mechanism for this substance would serve an important function.

Gershon and Ross studied in mice in vivo the binding and distribution of 5-HT after administering its radioactive precursor, 5-hydroxytryptophan (6, 7). They found that the lung was able to accumulate and retain 5-HT for long periods of time in cells which they identified as septal cells, or lung macrophages (7). Our findings do not contradict theirs, but simply indicate that there are at least two pools of 5-HT: one, that results from the rapid uptake of 5-HT in blood by pulmonary capillary endothelial cells, and another (in a different cell population), that results when 5-HT is formed within the tissue from 5-hydroxytryptophan.

Since this study has established that pulmonary endothelial cells are the site of 5-HT-3H uptake, and because this function is apparently not shared to a significant extent by any of the other cell populations of the lung, it is reasonable to infer that the mechanism of uptake of 5-HT previously described (12), namely a Na+-dependent, carrier-mediated transport, operates in pulmonary endothelial cells. Such a process has already been described in many other cellular systems (17), but, to the best of our knowledge, never in the lung or in endothelial cells. We also believe that these cells contain monoamine oxidase (MAO) in substantial amounts, although Tyler and Pearse, using a histochemical technique, concluded that only moderate amounts of MAO existed in pulmonary endothelial cells (26).

Our observations, however, leave an important question unanswered. Is the uptake of 5-HT a phenomenon specific for endothelial cells of the lung, or does it also occur in the vascular endothelium of other organs? Since no direct radioautographic or pharmacologic studies have been made on other organs, we may only speculate. In two experiments in which Thomas and Vane infused 5-HT into peripheral vascular beds, they reported that it was inactivated to some extent (25), but they did not investigate the nature of this process. Other investigators have studied the metabolism and uptake of 5-HT in the intestine (28), but their technique of using pieces of tissue is not suitable for the demonstration of a possible role played by endothelial cells. The lung is an ideal tissue for demonstrating this uptake phenomenon since its great number of capillaries provides a vast endothelial surface area in direct contact with the circulation. The fact that the lung is the only major organ that does not inactivate angiotensin II to a significant extent (10) suggests that pulmonary endothelial cells might have specific properties not shared by endothelial cells of other organs. Functions such as the hydrolysis of angiotensin I (14) and bradykinin (16), and the uptake and metabolism of adenosine triphosphate (19) and norepinephrine (11), have also been attributed to the endothelial cells of the lung, on the basis of indirect evidence or theoretical considerations. When isolated endothelial cells become available, these suppositions can be confirmed. However, in view of the present findings, pulmonary endothelial cells can no longer be considered to function primarily as a passive barrier between the air and the blood, since they play a major role in the uptake of 5-HT.

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