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# Preparation of a Human Lung Purified Plasma Membrane Fraction: Confirmation by Enzyme Markers, Electron Microscopy, and Histamine $H_1$ Receptor Binding

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**Summary.** A simple and rapid method of isolating plasma membranes from human peripheral lung tissue is described. The method involves homogenization of tissue in 0.25 M sucrose-buffered medium followed by differential and sucrose density gradient centrifugation. Enzymatic and morphological characterization of the plasma membrane fraction revealed minimal contamination by nonplasma membrane fragments. The isolated plasma membranes showed an 18-fold purification of 5′-nucleotidase activity compared to the original homogenate. Electron-microscopic studies of the plasma membrane fraction revealed the presence of small membrane vesicles having a trilaminar membrane structure. To further examine the purity of the plasma membrane preparation, the binding of the $H_1$ receptor antagonist, $^3H$ pyrilamine, to the plasma membrane-enriched fraction was compared to the binding to crude membrane preparations. Both the plasma membrane-enriched fraction and the crude membrane preparation had similar $K_d$'s for the histamine antagonist, but the plasma membrane-enriched fraction had a threefold greater binding capacity, reflecting the relative enrichment of plasma membranes of the preparation. Thus, a method has been developed for the isolation of plasma membranes from human peripheral lung which should provide material for a variety of biochemical and pharmacological studies.

**Key Words** plasma membrane · lung · histamine receptors · 5′nucleotidase

## Introduction

The human lung responds to a great variety of hormonal stimuli with biochemical and physiologic responses. For instance, changes in cyclic nucleotide levels [10–12, 17] and the generation of prostaglandins [17, 20] in intact human lung have been reported to occur as a result of immediate hypersensitivity reactions and hormonal stimuli. In order to extend these studies to radioligand and biochemical analyses of histaminergic, adrenergic, and cholinergic receptors so as to further define the pathophysiologic events underlying allergic asthma, it became necessary to develop a rapid, yet effective method to isolate human lung plasma membranes. Methods have previously been reported for isolation of plasma membrane from rat lung [16], mesenteric artery [14], myometrium [13] and liver [3], but to our knowledge no previous report of methods to isolate and characterize plasma membrane from human peripheral lung have been published. The method which has been developed employs simple techniques resulting in the isolation in less than 6 hr of a plasma membrane-enriched fraction (PMF) that is relatively free of contaminating organelles. The purity of the preparations was examined by a number of criteria including enzyme analyses, electron microscopy, and histamine $H_1$ receptor binding.

## Materials and Methods

### Reagents

Cytochrome *c* (Type VI), cytochrome *c* reductase, diphenhydramine, KCN, NADH, pyruvic acid, L-ascorbic acid, AMP, 5′nucleotidase, cytochrome *c* oxidase, $K_3Fe(CN)_6$, phenylmethylsulfonyl fluoride (PMSF) and sodium tartrate were purchased from Sigma Chemical Co., St. Louis, Mo.; sucrose was from Fisher Scientific Co., Fair Lawn, N.J.; ammonium molybdate was from Mallinckrodt Chemical Works, St. Louis, Mo.; bovine serum albumin (BSA) was from Miles Laboratories, Inc., Elkhart, Ind.; Tris was from Boehringer Mannheim, Indianapolis, Ind.; Hypaque-M, 90% (30% [wt/vol] of the sodium salt and 60% [wt/vol] of the meglumine salt of diatrizoic acid) was from Winthrop Laboratories, New York, N.Y.; $^3H$ pyrilamine (specific activity 27.3 Ci/mmol) was from New England Nuclear, Boston, Mass.; and Azocoll was from Calbiochem-Behring Corp., La Jolla, Calif.

### Human Lung

Human peripheral lung tissue free from obvious macroscopic pathology was taken from lung tissue removed at thoracotomy,

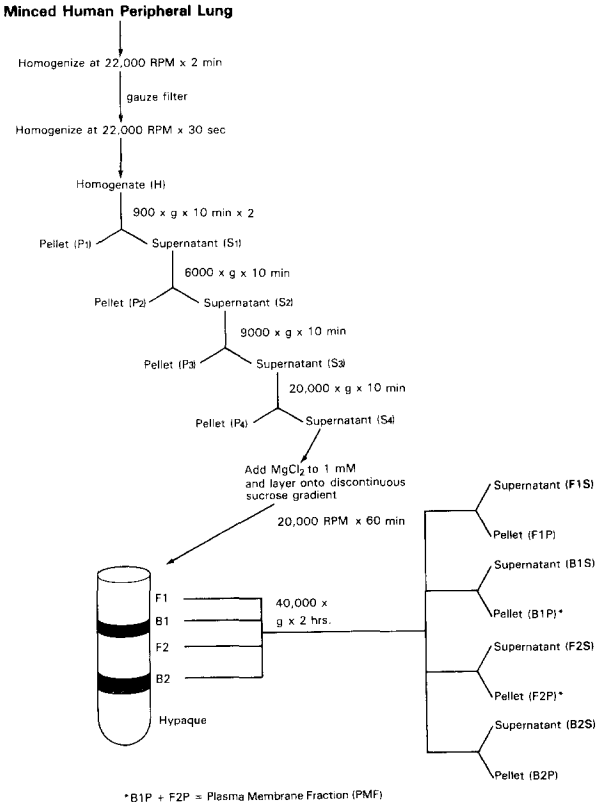


**Fig. 1.** Outline of isolation procedures for PMF

generally from patients with bronchial carcinoma. The lung samples were washed extensively in Tyrodes buffer and immediately frozen at −70°C until use.

## Preparation of PMF

Figure 1 represents an outline of the procedure, which was developed to isolate the PMF. All operations were performed at 0 to 4°C. Lung fragments were minced and placed in ice-cold 10 mM Tris containing 0.25 M sucrose, 0.5% BSA, and 0.5 mM PMSF. The lungs were homogenized using a Brinkmann Polytron PCU-2 homogenizer at 22,000 rpm for 2 min. The homogenate (H) was filtered through double-layered gauze cloth and then rehomogenized for 30 sec at 22,000 rpm.

The H was centrifuged at 900 × *g* × 10 min twice to remove residual tissue fragments, unbroken cells, nuclei, and large cellular debris (this fraction is designated P1). All subsequent centrifugations were carried out in a Dupont Sorvall RC-5B refrigerated superspeed centrifuge using an SS-34 angle rotor except for the discontinuous sucrose gradients for which an SV-288 vertical rotor was used. The supernatants from the initial centrifugations ($S_1$) were sequentially centrifuged for 10 min at 6,000, 9,000 and 20,000 × *g* (supernatants from which are designated $S_2$, $S_3$, and $S_4$). $MgCl_2$ was added to the final supernatant ($S_4$) to a 1 mM concentration in order to enhance separation of ribosomes and smooth endoplasmic reticulum membranes from the plasma membranes by sucrose density gradient centrifugation [16]. Eight ml of $S_4$ was applied to the top of a discontinuous sucrose gradient which was composed of 10 ml each of 7.5 and 15% sucrose and 5 ml of 90% Hypaque-M. Other gradient compositions were tested but were less efficient in isolating a plasma membrane-rich fraction. The gradient tubes were centrifuged for 60 min at 20,000 rpm (35,000 × *g*). Four distinct fractions in the sucrose gradient (Fig. 1) were identified and removed by Pasteur pipettes. Each of the four fractions was subsequently diluted 1 : 1 with 0.1 M Tris containing 0.5 mM PMSF and centrifuged at 40,000 × *g* for 2 hr. The four pellets (F1P, B1P, F2P, and B2P) were resuspended in Tris, and the B1P and F2P were combined into the plasma membrane fraction (PMF). Preliminary experiments indicated that the content of protein and marker enzymes for the B1P and F2P fractions were very similar and that these two fractions contained the greatest amount of specific 5′-nucleotidase activity. The B1P and F2P fractions also were similar by electron microscopy, and each fraction contained much more plasma membrane material than the other fractions. All fractions were maintained at −70°C until assayed for protein, enzyme activity, and histamine $H_1$ receptors.

## Protein Estimation

The protein content of the fractions was determined by the method of Lowry [15] using BSA as the standard.

## Enzyme Assays

Spectrophotometric measurements for all enzyme assays were performed on a Gilford Model 250 spectrophotometer. 5′-Nucleotidase, lactic dehydrogenase (LDH), cytochrome *c* oxidase, and cytochrome *c* reductase were used as markers for plasma membrane, cytosol, mitochondria and microsomes, respectively [9, 21].

5′-Nucleotidase was assayed at 37°C in 100 mM Tris buffer, pH 7.4, containing 10 mM $MgCl_2$ using 5 mM AMP as the substrate [19]. The inorganic phosphate released during the 60-min reaction time was determined by a slight modification of the Ames and Dubin technique [1] using an ascorbic acid-ammonium molybdate solution prepared in 0.75 N $H_2SO_4$. The activity was determined at 820 nM from a standard curve using purified 5′-nucleotidase.

LDH activity was determined at 22°C by following the rate of oxidation of NADH (8 mM) in 0.05 M phosphate-buffered saline (PBS) (pH 7.5) containing 0.31 mM pyruvate [2]. One unit of activity was defined as the change in absorbance at 366 nm/min × 10,000.

Cytochrome *c* oxidase activity was measured at 22°C using 34 μM reduced cytochrome *c* in 0.03 M PBS, pH 7.4. The rate of oxidation of cytochrome *c* was determined by measuring the decrease in absorbance at 550 nM [4].

One unit of activity was defined as:

$$\log \frac{(A_{t1} - A_{ox}) - \log(A_{t2} - A_{ox})}{t2 - t1}.$$

$A_{t1}$, $A_{t2}$ and $A_{ox}$ = absorbance at time $t_1$, $t_2$, and after the addition of $K_3Fe(CN)_6$, respectively.

Cytochrome *c* reductase activity was measured at 22°C using 0.2 M PBS (pH 7.4), 0.02% NADH, 1% cytochrome *c*, 5 mM KCN and enzyme in a 2-ml volume [5]. The reduction of cytochrome *c* was followed at 550 nM. The activity was determined from a standard curve using cytochrome *c* reductase obtained from Sigma Chemical Co.

### Protease Assays

One hundred-$\mu$l aliquots of the H and its supernatants were incubated at 37°C for 15 min with 1 ml of Azocoll solution containing 5 mg of Azocoll/ml of PBS, pH 7.0. The reaction was terminated by centrifugation at 400 × $g$ for 3 min. Protease activity was determined by following the absorbancy at 520 nM of the supernatants, which contain the soluble products generated during Azocoll degradation, and comparing these results to standard curves developed with specific proteases.

### Electron Microscopy

The PMF was pelleted for 2 hr at 40,000 × $g$, fixed in 1% glutaraldehyde in PBS (pH 7.2), washed in 0.1 M cacodylate buffer, post-fixed in 2% cacodylate-buffered osmium tetroxide, and embedded in Maraglas®. Ultrathin (silver-grey) sections were cut on an LKB ultratome IV using a Diatome diamond knife, stained with uranyl magnesium acetate and lead citrate, and examined in a Hitachi-Hu-11E electron microscope at 75 kV.

### Histamine $H_1$ Receptor Radioligand Binding Assays

$^3$H Pyrilamine was used to radiolabel $H_1$ receptors located on the plasma membrane [6, 18]. PMF or $S_1$ (also designated as crude membrane preparations, CMP) were suspended in 0.125 M Tris buffer containing 0.025 M $MgCl_2$ (pH 7.4) in a final concentration of 2 mg/ml of protein. One hundred-$\mu$l aliquots of either PMF or CMP suspensions were incubated at 22°C for 30 min with the $H_1$ antagonist, $^3$H pyrilamine (5 to 120 nM), in the presence or absence of 100 $\mu$M diphenhydramine ($H_1$ antagonist) for determination of nonspecific binding. Incubations were terminated by adding 4 ml of ice-cold Tris buffer followed by rapid vacuum filtration of the samples through diphenhydramine-presoaked Whatman GF/C glass filters. The filters were washed immediately thereafter with 24 ml of ice-cold buffer, dried, and then assayed in a liquid scintillation system using a Beckman LS-9000 counter. All samples were run in duplicate, and the replicates differed from each other by less than 10%. Specific binding of ligand to membrane suspensions, defined as total binding minus nonspecific binding, was 60 to 70% at subsaturating $^3$H pyrilamine concentrations.

## Results

### Protein Analysis

The protein content of the subcellular fractions prepared from human peripheral lung by the scheme illustrated in Fig. 1 is listed in Table 1. The PMF (B1P, F2P) accounted for 0.4% of the total protein in the H.

**Table 1.** Protein content of the fractions[a]

| Fractions ($n$ = 4) | Protein $\left(\frac{\text{mg}}{\text{g starting lung}}\right)$ | | Recovery (%) |
|---|---|---|---|
| H | 119.42 ± 17.85 | | 100.0 |
| P1 | 21.01 ± 2.36 | | 17.6 |
| P2 | 5.03 ± 0.91 | | 4.2 |
| P3 | 3.13 ± 0.25 | | 2.6 |
| P4 | 2.99 ± 0.91 | | 2.5 |
| F1S | 32.81 ± 8.84 | | 27.5 |
| F1P | 0.38 ± 0.05 | | 0.3 |
| B1S | 6.80 ± 0.60 | | 5.7 |
| F2S | 2.46 ± 1.33 | | 2.1 |
| PMF | 0.44 ± 0.09 | | 0.4 |
| B2S | 12.36 ± 3.04 | | 10.4 |
| B2P | 0.74 ± 0.24 | | 0.6 |
| | | (Total) | 73.9 |

[a] Protein content expressed in mg/g of starting lung tissue. Subcellular fractions were prepared as in Fig. 1. The results represent the $\bar{X}$ ± SEM of four lung preparations.

### Enzyme Analysis

The enzymatic activities of several preparations obtained from five different human lungs are documented in Table 2. 5′-Nucleotidase specific activity was 18 times greater in the PMF as compared to the H, indicating a marked enrichment of this activity in the PMF. Every fraction except for B2P had a 5′-nucleotidase specific activity that was 8- to 80-fold less than that in the PMF. Analysis of the B1P and F2P fractions separately and combined gave similar results.

LDH activity as a marker of soluble cytosolic components was present in the PMF, but in a much lower quantity than in most other fractions. The specific activity of LDH in the PMF was approximately 60% of that noted in the H, and the PMF contained only 0.2% of the total activity found in all the fractions. The highest specific activity was in the F2S fraction.

Fifty-five percent of the cytochrome $c$ oxidase activity was present in the first three pellets, while the lowest percentages were in the PMF and F1P fractions (0.6 and 0.5%, respectively). The highest specific activity of cytochrome $c$ oxidase was in the F2S fraction.

In spite of the considerable enrichment with respect to plasma membrane, all of the preparations showed appreciable, though variable, contamination with microsomal (cytochrome $c$ reductase) material.

### Protease Assays

There were no significant changes in the $Å_{520}$ of the samples assayed. Thus, by the Azocoll technique, no protease activity in the H or supernatants could

**Table 2.** Enzymatic activities of the fractions[a]

| Fractions | 5′-Nucleotidase | | LDH | | Cyt. *c* oxidase | | Cyt. *c* reductase | |
|---|---|---|---|---|---|---|---|---|
| | Activity $\left(\frac{\text{milliunits}}{\text{mg protein}}\right)$ | Recovery (%) | Activity $\left(\frac{\text{units}}{\text{mg protein}}\right)$ | Recovery (%) | Activity $\left(\frac{\text{milliunits}}{\text{mg protein}}\right)$ | Recovery (%) | Activity $\left(\frac{\text{units}}{\text{mg protein}}\right)$ | Recovery (%) |
| H | 8.95 ± 2.96(3) | 100.0 | 92.06 ± 6.94(4) | 100.0 | 1.44 ± 0.44(4) | 100.0 | 0.44 ± 0.13(5) | 100.0 |
| P1 | 13.26 ± 3.67(5) | 26.1 | 103.94 ± 12.01(3) | 19.9 | 2.89 ± 1.52(4) | 35.3 | 0.15 ± 0.02(4) | 6.0 |
| P2 | 8.68 ± 1.56(4) | 4.1 | 78.44 ± 10.75(4) | 3.6 | 4.84 ± 1.17(4) | 14.2 | 0.66 ± 0.16(5) | 6.3 |
| P3 | 20.77 ± 6.53(5) | 6.1 | 118.08 ± 8.80(4) | 3.4 | 2.99 ± 0.31(4) | 5.4 | 1.74 ± 0.77(5) | 10.3 |
| P4 | 16.83 ± 6.73(5) | 4.7 | 79.47 ± 14.26(4) | 2.2 | 2.34 ± 0.29(4) | 4.1 | 1.19 ± 0.18(4) | 6.8 |
| F1S | 13.68 ± 0.81(4) | 42.0 | 125.23 ± 14.88(4) | 37.4 | 0.33 ± 0.23(4) | 6.3 | 0.04 ± 0.02(5) | 2.5 |
| F1P | 15.47 ± 9.18(4) | 0.6 | 104.24 ± 23.57(4) | 0.4 | 2.08 ± 1.26(4) | 0.5 | 0.35 ± 0.18(5) | 0.3 |
| B1S | 15.85 ± 3.71(4) | 10.1 | 133.55 ± 25.99(3) | 8.3 | 0.70 ± 0.36(3) | 2.8 | 0.43 ± 0.07(4) | 5.6 |
| F2S | 8.72 ± 8.72(4) | 2.0 | 293.31 ± 117.24(4) | 6.6 | 6.16 ± 3.62(4) | 8.8 | 0.60 ± 0.19(4) | 2.8 |
| PMF | 157.35 ± 48.80(5) | 6.5 | 58.65 ± 7.23(4) | 0.2 | 2.43 ± 0.75(4) | 0.6 | 3.73 ± 1.04(5) | 3.1 |
| B2S | 1.96 ± 1.36(5) | 2.3 | 20.90 ± 4.98(4) | 2.3 | 1.18 ± 0.17(3) | 8.5 | 0.08 ± 0.04(4) | 1.9 |
| B2P | 77.89 ± 23.01(5) | 5.4 | 31.78 ± 8.97(4) | 0.2 | 3.36 ± 0.98(4) | 1.4 | 2.83 ± 0.55(5) | 4.0 |
| Total Recovery (%) | | 109.9 | | 84.5 | | 87.9 | | 49.6 |

[a] Distribution of enzyme activities in subcellular fractions prepared from human peripheral lung by the scheme described in the text. The results are expressed as mean ± SEM from the number (*n*) of experiments shown in parentheses. *See* the text for the definition of specific enzyme activities.

be detected, probably indicating that the BSA that was added to the isolation buffer to stabilize lysosomal membranes [14] and the PMSF added as a protease inhibitor [8] prevented protease activity from occurring in the samples.

### Electron Microscopy

The PMF, as examined by electron microscopy, consisted chiefly of small membrane vesicles (Fig. 2). Some larger vesicles and linear membrane profiles were also present, as were occasional electron-dense myelin figures. The inset in Fig. 2 demonstrates the trilaminar membrane profiles that were found both on the vesicles and linear membrane fragments.

### Histamine $H_1$ Receptor Assays

The CMP ($S_1$) and PMF were examined for the presence of histamine $H_1$ receptors by radioligand binding techniques. Increasing concentrations of $^3$H pyrilamine were incubated with samples of CMP and PMF. Specific binding of $^3$H pyrilamine was saturable with a $K_d$ of 102 nM for the CMP and 110 nM for the PMF. Scatchard analysis of a representative experiment comparing the CMP and the PMF is shown in Fig. 3. The linear plots suggest one class of binding sites. From these Scatchard plots, a maximum binding capacity of 125 fmol/mg of protein and 370 fmol/mg of protein were calculated for the CMP and PMF, respectively. Since the CMP and the PMF have similar $K_d$'s for $^3$H pyrilamine, the presence of a threefold greater binding capacity in the PMF indicates the relative enrichment of plasma membrane in the preparation.

## Discussion

Isolation of purified plasma membranes from human peripheral lung tissue is inherently difficult for several reasons. First, peripheral lung is composed of many cell types (e.g., smooth muscle cells, endothelial cells, fibroblasts, epithelial cells, Clara cells, macrophages, mast cells, and leukocytes). The membranes obtained from these various cell types are invariably contaminated because of the difficulty in separating vesiculated membranes and organelles having an array of different densities and sizes. Second, peripheral lung tissue is always fibrous, thereby preventing gentle homogenization from disrupting the majority of cells. Vigorous homogenization results in the generation of small fragments of plasma membrane which may be lost at different centrifugation speeds. This may in part account for the finding of 5′-nucleotidase activity in fractions other than the PMF. Third, the multiple-step procedure outlined on Fig. 1 must result in some contamination because of entrapment of other vesicles and cytosol into the membrane vesicles and

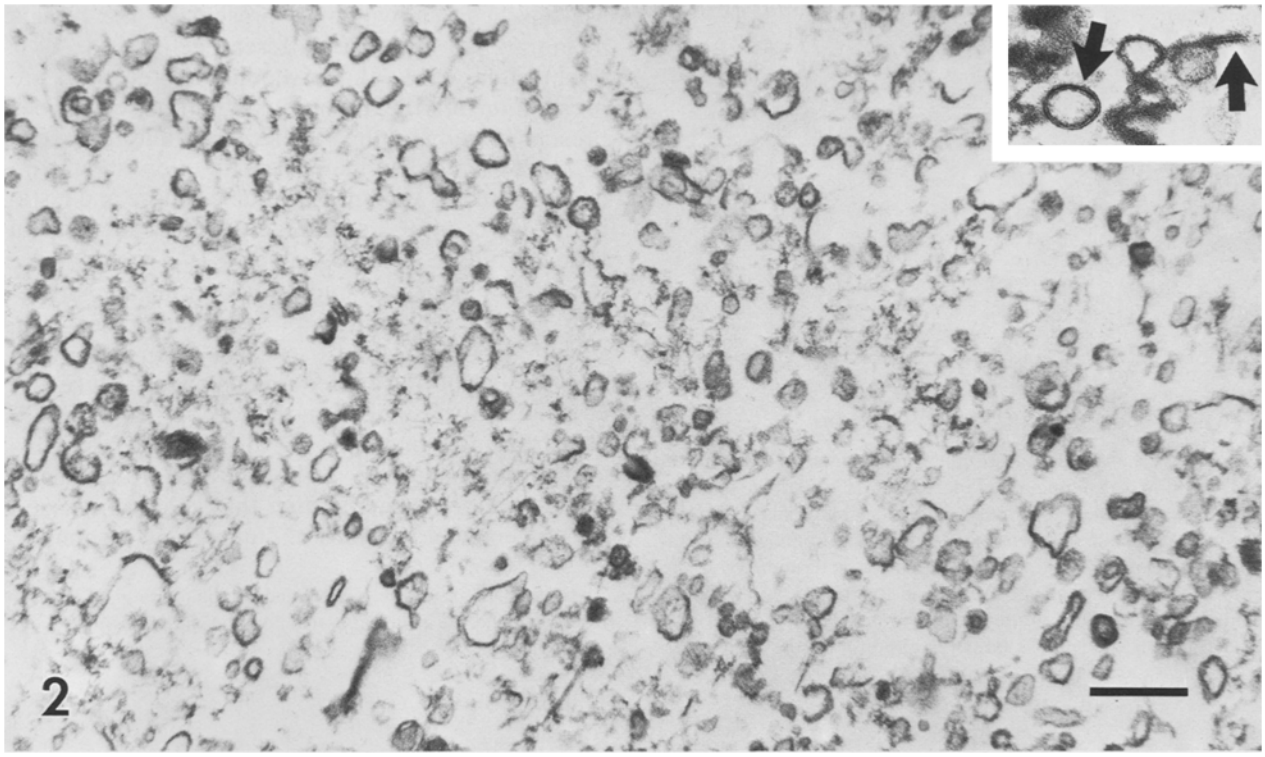


**Fig. 2.** Electron micrograph of an ultrathin section prepared from the PMF showing numerous small membrane vesicles (61,500×). Scale marker = 0.2 $\mu$m. Inset: Trilaminar membrane structures indicated by arrows (85,000×)

inversion of membranes during vesiculation to give right-side-out and inside-out vesicles [9].

Since each subcellular organelle has a characteristic set of functions, it is implicit that at least some of the functional components are qualitatively unique for each. Thus, the presence of characteristic enzymes and receptor binding sites provides a means of identifying membrane fractions. Ideally, for a constituent to serve as a marker it must be confined to only one class of organelles and be present in every organelle of that particular class [9]. Since the above conditions are rarely fulfilled by any single marker, both marker enzymes and hormone receptor binding were employed to identify the PMF.

5′-Nucleotidase is often used as a plasma membrane marker enzyme [3, 9, 13, 14, 16, 19]. Although it is doubtful that 5′-nucleotidase is exclusively present in the plasma membrane, it is reported to be considerably enriched in PMF isolated from different sources [13]. The isolated PMF had 18 times greater specific activity of 5′-nucleotidase as compared to crude lung H. Similar techniques employed to isolate plasma membranes from rat lung [16] and rat artery [14] demonstrate a six- to eightfold enrichment of 5′-nucleotidase. Some activity was present in other fractions as well, an observation which could reflect the presence of contaminating plasma membrane or the localization of 5′-nucleotidase to other subcellular structures [13].

In addition to the enzymes enumerated in the results, glucose-6-phosphatase activity was measured, and none was found in the H or other fractions. Therefore, this enzyme cannot be used as a marker for microsomes (endoplasmic reticulum) in human lung. Our results are similar to those found in rat lung, which also has very little pulmonary glucose-6-phosphatase activity [7].

The purity of the PMF was analyzed by morphological criteria as well as enzyme analysis. Electron-micrographic analysis of the PMF (Fig. 2) showed homogenous membranes that were essentially free of contamination by other recognizable subcellular components. At higher magnification (Fig. 2, inset) a characteristic triple layer of membrane was evident in vesicles and in linear membrane profiles.

Plasma membranes may contain specific receptors for selected hormones, which may be measured by the use of radioligand binding techniques. Employing $^3$H pyrilamine, an $H_1$ receptor antagonist, as the radioligand, and diphenhydramine, another

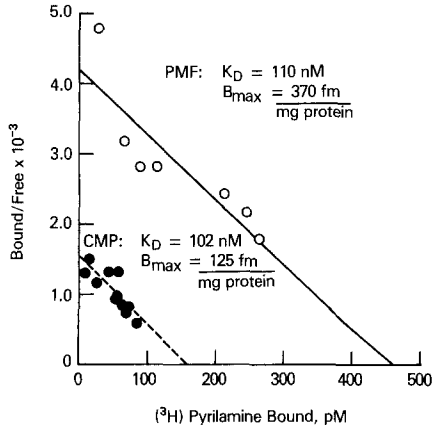


**Fig. 3.** Scatchard plots of specific binding of $^3$H pyrilamine to CMP and PMF as a function of increasing $^3$H pyrilamine binding. Binding was carried out as described under Materials and Methods in the absence and presence of diphenhydramine to saturation. Each point is the average of duplicate determinations. The ratio of specific bound $^3$H pyrilamine to free $^3$H pyrilamine is plotted as a function of specifically bound $^3$H pyrilamine. The straight line is the least-squares regression fit of these data

chemically distinct $H_1$ receptor antagonist, as the competitive displacing agent, we have identified and characterized $H_1$ receptors in human lung tissue. The binding of $^3$H pyrilamine to sites on human lung plasma membrane is specific, rapid, reversible, saturable and stereoselective (*unpublished data*). Using these techniques, the radioligand binding capacities of the PMF and CMP were compared. These data indicate that purification of plasma membranes containing histamine receptors does not affect the chemical interaction between the receptor and ligand (the $K_d$) but does increase the relative binding capacity of the preparation (the $B_{max}$). This observation supports the use of crude membrane preparations for assessment of receptor affinity but introduces some caution in accepting the validity of binding sites determinations in crude preparations. On the other hand, maintenance of receptor-ligand affinity in highly enriched plasma membranes suggests that the isolation procedure has not altered the membrane configuration or structure in a major way and adds further support to the effectiveness of the preparation.

The $S_1$ was analyzed for 5′-nucleotidase and was found to contain 11.56 ± 5.11 mu/mg of protein ($n$ = 5). Thus, the value of 157.35 ± 48.80 mu/mg of protein ($n$ = 5) corresponds to an enrichment factor of approximately 13 for the PMF. However, the H1 receptor binding assay only indicates a threefold purification. This apparent discrepancy between the 5′-nucleotidase and H1 receptor binding assays is not unexpected. As stated previously, 5′-nucleotidase has been shown to be present on the plasma membrane of multiple different cell types. It is doubtful, however, that histamine H1 receptors are present on all the numerous different cell types present in human lung. Therefore, while the purification of a plasma membrane fraction should increase both the amount of 5′-nucleotidase and histamine H1 receptors, a relatively greater increase in 5′-nucleotidase is to be expected.

In summary, a rapid and reproducible procedure for the isolation of plasma membranes from peripheral human lung tissue has been described. The validity of this procedure has been analyzed by enzyme analyses, electron microscopy, and radioligand receptor analyses. By all three criteria, this procedure results in a highly purified, functionally intact membrane preparation that should be useful for biochemical analyses of human lung responses.

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