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TITLE Analysis of Proteins in Bronchoalveolar Lavage Fluids During Pulmonary Edema Resulting from Nitrogen Dioxide and Cadmium Exposure

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# ANALYSIS OF PROTEINS IN BRONCHOALVEOLAR LAVAGE FLUIDS DURING PULMONARY EDEMA RESULTING FROM NITROGEN DIOXIDE AND CADMIUM EXPOSURE

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## Summary

We have developed a new HPLC method by which quantitative measurements can be made on the biochemical constituents of the extracellular fluid lining of the lung as sampled by bronchoalveolar lavage. Nine of the fractions are proteins, two are phospholipids, and two fractions remained unidentified. Rats were subjected to the intrapulmonary deposition of cadmium, a treatment model known to induce pulmonary edema and cause a translocation of blood compartment proteins into the lung's alveolar space compartment. Resulting pulmonary edema was hallmarked by ~25-fold increases in three major blood compartment-derived HPLC protein fractions, two of which have been identified as albumin and immunoglobulin(s). Analysis of lavge fluid from rats exposed to 100 ppm $NO_2$ for 15 min, an exposure regimen which also produces pulmonary edema, indicated that the three blood compartment proteins in the lavage fluids were elevated 35- to 72-fold over controls 24 h after exposure. These results demonstrate that HPLC can be used to provide a highly sensitive method for detection and quantitation of pulmonary edema that can occur in acute lung injuries resulting from environmental insults. (This work was conducted under the auspices of the U.S. Dept. of Energy).

## Introduction

An extracellular lining fluid (ELF) covers the epithelium of the alveoli and conducting airways of the lung. This ELF contains a complex mixture of lipids and proteins, inter alia (1). While extensive work has focused on the phospholipid components in ELF, the protein constituents are much less characterized in terms of their origin, biochemical characteristics, and their significance in physiologic and pathophysiologic processes. In order to study these proteins, our laboratory has developed a high-performance liquid chromatography (HPLC) method for fractionating ELF proteins (2,3), as sampled by relatively non-invasive bronchoalveolar lavage (4). In this report, we demonstrate applications of the HPLC method for detecting and quantifying protein alterations in ELF that occur during acute lung injury in which permeability pulmonary edema is a prominent pathologic component.

## Qualitative Analysis of the HPLC Fractions

Since lung lavage is a method by which ELF can be sampled, we based our HPLC method on the adsorption of proteins from bronchoalveolar lavage fluid (BALF) (5) onto a C18 reversed phase chromatography column. Six fractions were then eluted from the column using a series of acetonitrile gradients and isocratic steps that progress from $H_2O$ containing 0.2% trifluoroacetic acid (TFA) to 65% $CH_3CN$/0.2% TFA. Following this, five additional fractions were eluted with methanol (Fig. 1). Constituents in these fractions can be recovered by lyophilization, since all of the HPLC solvents are volatile (6). All eleven fractions can be detected by monitoring the column effluent at 206 nm (Fig. 1A), but only nine can be detected at 280 nm. Details of the development of this procedure have been documented elsewhere (2,3).

Phospholipid analysis of the HPLC effluent was accomplished by collecting samples in a fraction collector and extracting them with chloroform-methanol (7) as described by the Fiske-Subbarow procedure (8) and modified by Shin (9) (Fig. 1B). Protein analysis was performed in similarly collected samples by

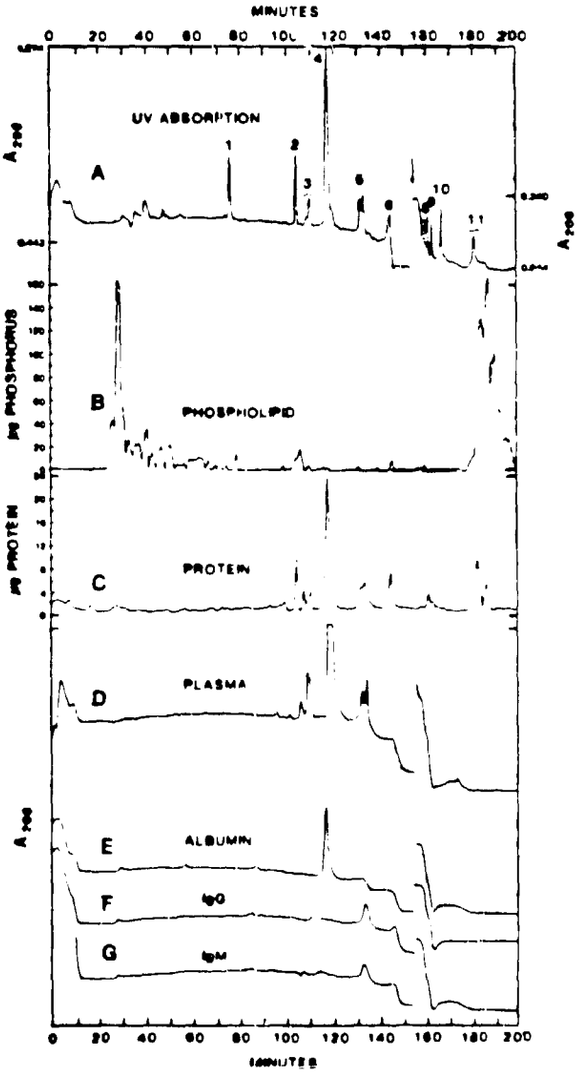


**Figure 1. Fractionation of BALF by HPLC.** Rat lungs were lavaged with 6 sequential washes of 8 ml phosphate buffered saline recovering 97% of the volume. The lung free cells were removed by centrifugation at 300 g and any remaining acellular solid material was removed by centrifugation at 2300 g for 10 min. Samples were stored at -70°C. Prior to HPLC, samples were made 0.2% with respect to TFA, which normally clarified any cloudiness. These samples were then loaded directly on a reversed-phase $\mu$ Bondapak C18 Radial-PAK cartridge without further treatment. HPLC elution was accomplished as described previously. (A) UV absorption profile of rat BALF monitored at 206 nm. (B) Phospholipid profile of rat BALF; (C) protein profile of rat BALF. (D) UV absorption profile of rat plasma. (E) UV absorption profile of rat albumin. (F) UV absorption profile of rabbit polyclonal IgG. (G) UV absorption profile of rabbit polyclonal IgM.

the Folin-Lowry procedure (10) (Fig. 1C). From these analyses, we found that phospholipids eluted before fraction 1 and also in the trailing shoulder of fraction 11. Therefore, by monitoring the HPLC at 206 nm, we are not measuring phospholipids. Proteins eluted in fractions 2-9 and 11. No protein was detected in fractions 1 and 10. These experiments indicated that ELF protein fractionation and analysis can be obtained from whole BALF without the need for intervening steps to remove phospholipids.

The proteins of ELF are derived from two sources with the larger quantity coming from the blood and the lesser amount from the cells of the lung itself. To determine which HPLC fractions might be blood proteins, comparisons were made between the elution profiles of rat lung lavage fluid, rat plasma, and certain protein standards. It was found that plasma proteins only eluted in HPLC fractions 3, 4 and 5 (Fig. 1D). Albumin eluted only in fraction 4 (Fig. 1E). Immunoglobulins G (Fig. 1F) and M (Fig. 1G) eluted primarily in fraction 5 with a small amount also occurring in fraction 6. The identification of fraction 4 as albumin was confirmed by treating BALF with anti-rat albumin IgG conjugated to Sepharose. Fraction 4 was essentially all removed from the BALF by this treatment. These results suggested that while fractions 3, 4 and 5 were derived from the blood compartment, fractions 1, 2, and 6-11 probably were derived from the lung cells themselves. Therefore, this fractionation procedure should be useful for distinguishing between injurious effects on the lung's cells and the resulting changes in the permeability status of the air-blood barrier resulting from environmental insults.

Quantitative Analysis of the HPLC Reactions

For this HPLC method to be useful for investigating protein changes during lung injury, it is most important that the quantitative response of each fraction be linear with respect to the amount of lavage loaded on the column. In Fig. 2, we demonstrate that the total amount of material eluted is proportional to the volume of BALF loaded on the column (Fig. 2A) and that the relationship between sample load and quantity of each fraction was linear (Fig. 2B). The regression coefficients (r) were >0.85 for all fractions, indicating this method can be used to detect quantitative changes in BALF components over a wide range of concentrations.

Cadmium Model of Pulmonary Edema

The heavy metal, cadmium (Cd), can cause an acute inflammatory response in the lung (11) and bring about changes in the lung's air-blood barrier that result in permeability pulmonary edema (12). During such injury, an abnormal abundance of blood proteins in BALF would be expected due to protein translocations from the lung's vasculature into the air space. In order to confirm that the HPLC fractions 3, 4, and 5 represent protein constituents common to blood, rats were intratracheally instilled with 7 $\mu$g of cadmium as $CdCl_2$ and lavaged (5) 24 h later. The BALF was subjected to HPLC and the fractions quantified (Fig. 3). Fractions 3, 4, and 5 comparably increased 25-, 27-, and 21-fold, respectively. This rapid and marked increase in these three fractions pointed to the blood compartment as their primary source, as was suggested by the experiments with standards in Fig. 1D).

Fractions 7 and 8 increased 8-fold (Fig. 3), making it impossible to resolve the two (Fig. 4C). Fractions 1, 6, 9, 10, and 11 increased about 2-fold (Fig. 3). The much smaller increases in these fractions (compared to the large increases in fractions 3, 4, and 5) suggest they are derived from an extra-vascular source, such as the lung tissue cells and/or lung matrix constituents. Concurrent with these general increases in BALF consitutents, fraction 2 was decreased by Cd treatment. Additionally, two new fractions (A and B) were observed in BALF during the inflammatory response (Fig. 3 and 4C).

The source of A and B may be blood; there were low levels of fractions eluting similarly to A and B in the plasma standard (Fig. 1D). However, this remains to be confirmed.

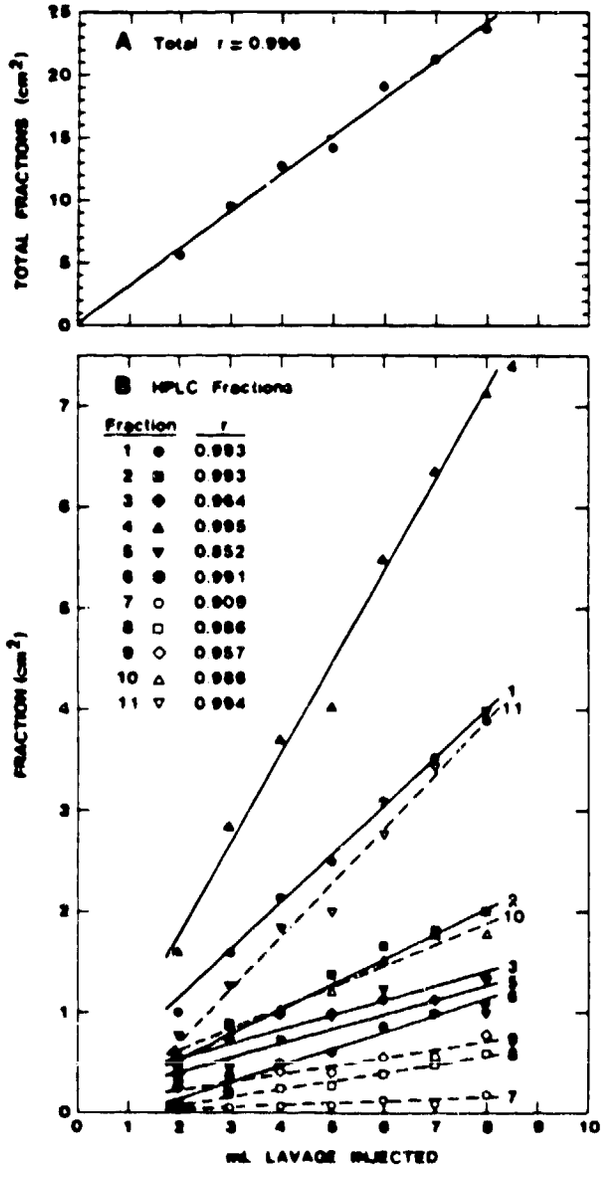


**Figure 2. Linear relationship between sample load and quantity of each fraction recovered.** Volumes of rat lung lavage ranging from 2 ml to 8 ml were made 0.2% with respect to TFA and pumped through the column at 1 ml/min. Following elution, each fraction was quantified by measuring the area of the eluted peaks detected by UV absorption at 206 nm. (A) The total amount of all eleven fractions recovered from the column. (B) The amount of each of the eleven fractions recovered from the column. The correlation coefficients (r) between volume loads and the areas of the peaks are shown on the figure. All "r" values are significant, $p < 0.05$.

Solubilization of BALF

The treatment of lungs with agents that cause an inflammatory response can result in lavage fluid that is cloudy. This cloudiness may be caused by the proteinaceous fluid and fibrin commonly observed histologically in the alveolar space following acute lung injury, like that produced by Cd. This proteinaceous material is no longer visible in the lung following lavage, indicating it has been successfully transferred to the BALF. The addition of TFA (which is a protein solubilizing agent in the HPLC system) to the lavage fluid clarified the BALF from Cd treated rats. Therefore, there were no particulate problems with insoluble samples in the HPLC analyses in Fig. 3. However, TFA alone was not sufficient to clarify the BALF from rats exposed to the oxidant

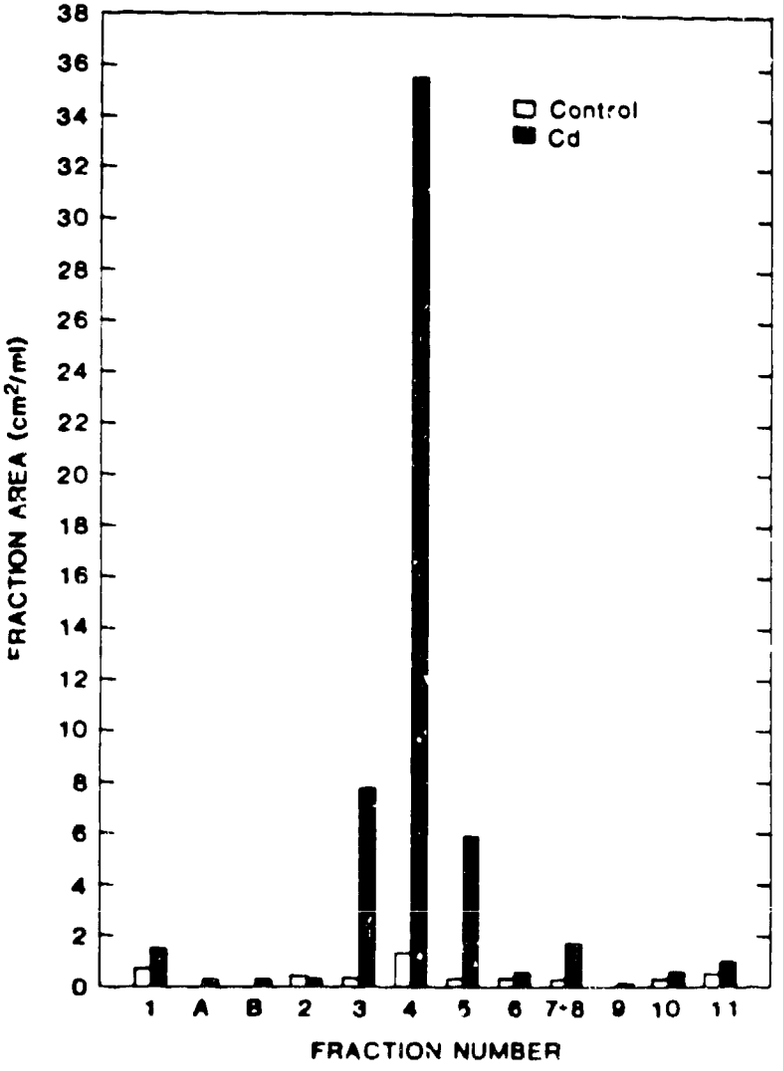


**Figure 3. Quantification of the HPLC profile of rat BALF following instillation of $CdCl_2$.** Each fraction was quantified by measuring the area under the peak and normalized to the volume of lavage loaded on the column. Due to the large differences in the quantities of the various fractions in cadmium treated rats, HPLC peaks 3, 4, and 5 were measured from small lavage loads (0.2 ml) while peaks 1, 2, 6-11, and A and B were measured from larger lavage loads (1.0 ml). Measurements of peaks from untreated control animals were made on 5 ml lavage loads. Each fraction represents the mean value of four animals.

gas, nitrogen dioxide (to be discussed). From previous experience, we knew that the use of 6 M guanidine hydrochloride to dissolve insoluble protein complex samples did not interfere with reversed-phase HPLC systems (13). Similarly, we found that BALF from $NO_2$ treated rats could be clarified by making the sample 6 M in guanidine·HCL.

To determine whether guanidine solubilization would cause any changes in HPLC profiles, comparisons were made between the profiles of untreated BALF and guanidine-treated BALF from control rats and similar BALF samples from rats exposed to Cd (Fig. 4). We found that addition of guanidine to BALF did not quantitatively change the HPLC profile in control rats (Fig. 4A and 4B), nor did it change the HPLC profile of BALF from Cd injured lungs (Fig. 4C). Thus, it was concluded that the clarification of BALF samples with 6 M guanidine·HCl could be used to solubilize constituents lavaged from extensively injured lungs in which alveolar flooding due to permeability pulmonary edema is substantial.

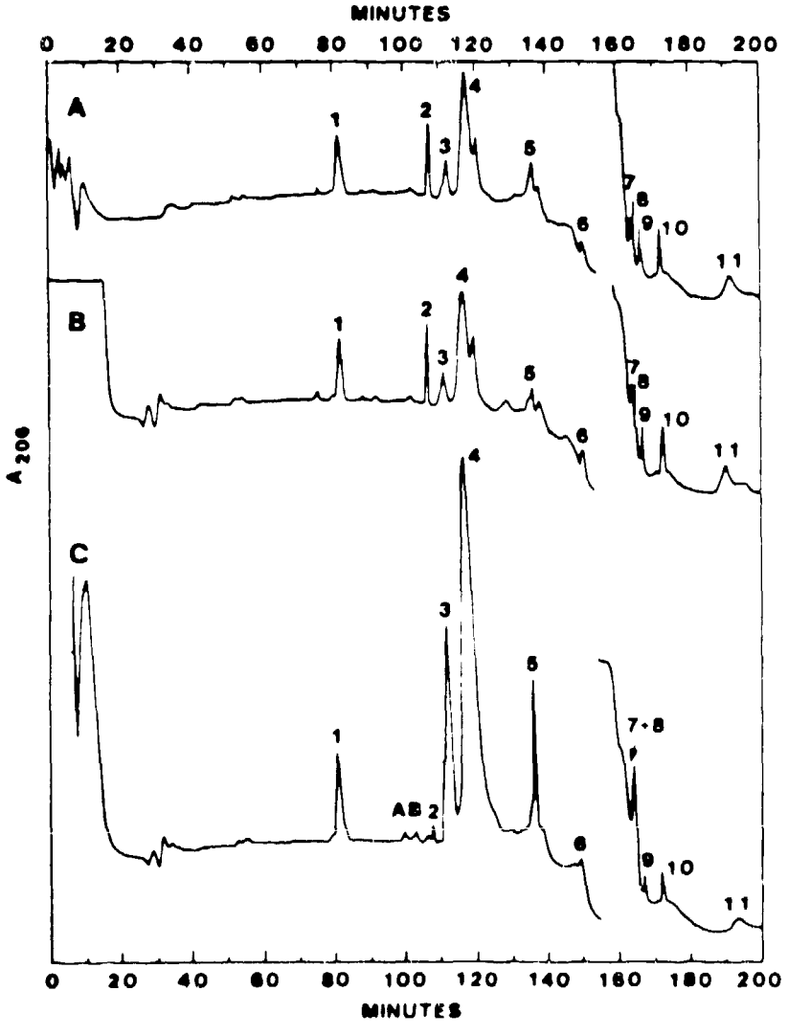


**Figure 4. HPLC of BALF solubilized with guanidine.** BALF samples were prepared for HPLC with 0.2% TFA as described in Fig. 1. Solid guanidine hydrochloride was added to these samples to a concentration of 6 M. These samples were loaded directly on the column. The guanidine was eluted in the void volume. (A) BALF not treated with guanidine from control rats. (B) BALF treated with guanidine from control rats. (C) BALF treated with guanidine from rats with cadmium-induced lung injury.

Nitrogen Dioxide Produced Pulmonary Edema

$NO_2$ is a potential health hazard in occupational settings where thermal combustion processes occur. For this reason, our laboratory has pursued a program that explores the physiological and biochemical results of lung exposure to this agent. In the course of these experiments, we have described increases in lung water and histopathologic changes; i.e., fibrin accumulation in peri-terminal bronchiolar-alveolar region, following $NO_2$ exposure (14,15) that are hallmarks of pulmonary edema. To determine the effects of $NO_2$-induced injury on the protein constituents in BALF, we quantified the HPLC fractions in BALF taken from rats 24 h after exposure to 100 ppm $NO_2$ for 15 min. BALF was clarified by adding guanidine·HCl to a 6 M concentration and then injected into the HPLC (Fig. 5). As expected, $NO_2$ exposure produced a major translocation of blood proteins to the lung's bronchoalveolar compartment. The amounts of these proteins (fractions 3, 4, and 5) were too high to quantify when normal control-level loads were injected (Fig. 5A). Therefore, it was necessary to inject very small loads in order to measure the blood proteins (Fig. 5B).

This quantification indicated that the blood-derived proteins, fractions 3, 4, and 5 increased 72-fold, 58-fold, and 35-fold, respectively (Fig. 6). Fractions 7 and 8 increased 8-fold (as it did following Cd exposure). Fraction 2 increased 6-fold (unlike its loss following Cd exposure). Fractions 2, 10, and 11 increased ~1.5-fold, but fraction 9 decreased to 35% of control (unlike its

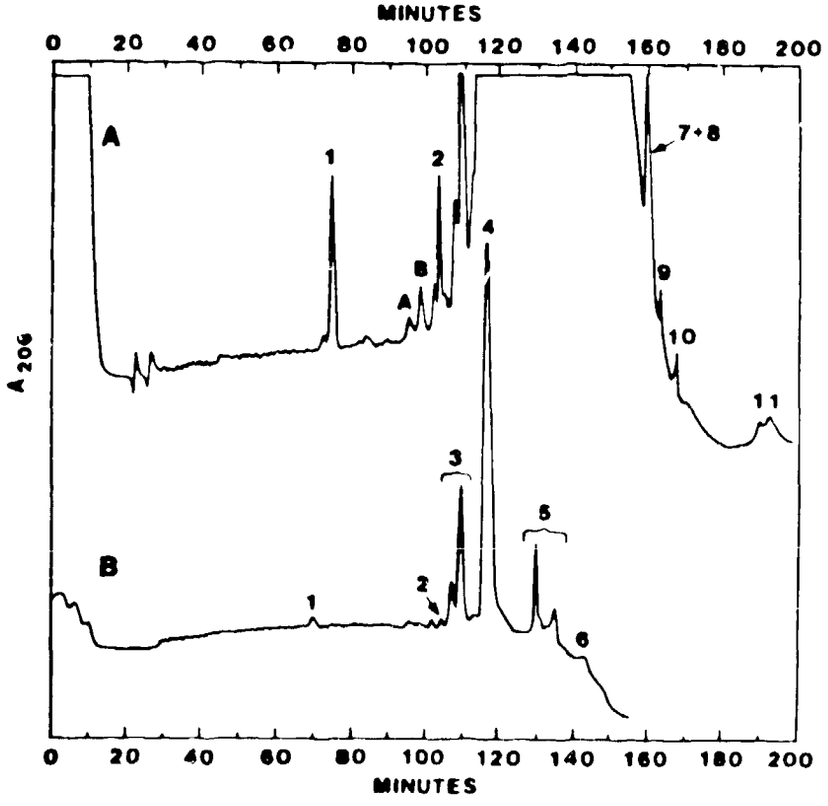


**Figure 5. HPLC of BALF from rats treated with $NO_2$.** Rats were exposed to 100 ppm $NO_2$ for 15 min and lavaged 24 h later. The cloudy BALF was clarified by a adding 6 M guanidine hydrochloride as described in Fig. 4. HPLC was run on (A) normal loads (3.5 ml of lavage fluid) and (B) small loads (0.2 ml of lavage fluid).

increase following Cd exposure). The fractions A and B were also observed following $NO_2$ exposure, as they were in the Cd-treated lungs, except the amount was greater after $NO_2$ exposure. This again suggests fractions A and B may be translocated from the lung's vascular compartment.

While most of the components of BALF responded to $NO_2$ exposure as they did in the Cd model, there was one outstanding difference between the two models of injury that may be of importance. Whereas the relative increases in the three major blood fractions were virtually identical in the Cd model, the relative increase in fraction 3 was approximately two times greater than the increases in the other two major blood fractions in the $NO_2$ model. Such findings suggest that the underlying permeability disturbance(s) involved in $NO_2$-induced pulmonary edema fundamentally differs from that produced by Cd. Further interpretations regarding this difference obviously requires the future identification of fraction 3. The comparison of these two models illustrate, nevertheless, that the use of HPLC to analyze protein constituents in BALF will offer significant utility in elucidating mechanisms at play in a variety of lung disorders in addition to pulmonary edema.

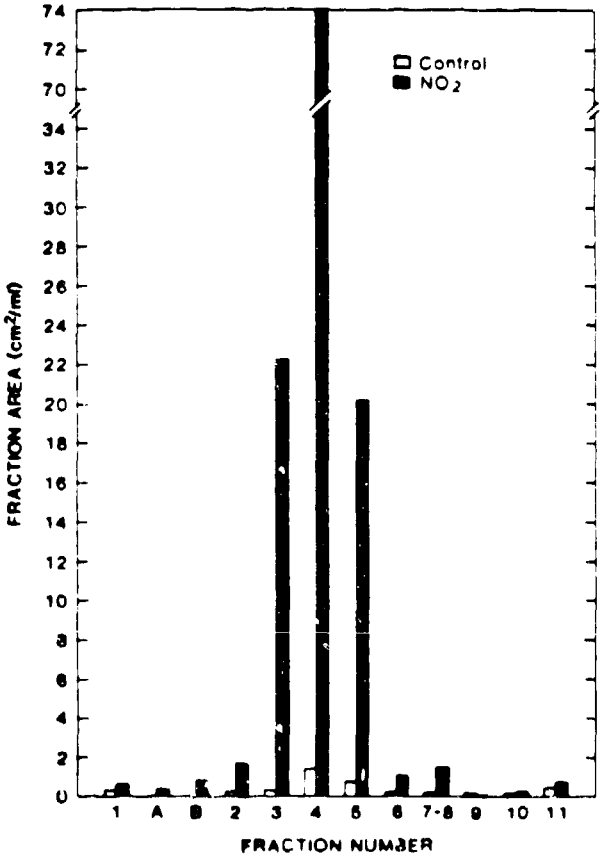


**Figure 6. Quantification of the HPLC profile of rat BALF following exposure to $NO_2$.** Each fraction was quantified as described in Fig. 3. Peaks 3, 4, 5, and 6 were measured from small loads (0.2 ml) and peaks 1, 2, 7-11, and A and B were measured from larger loads (3.5 ml). Measurements of the peaks from untreated control animals were made on 4 ml lavage loads. Each fraction represents the mean value of three animals for the $NO_2$ exposed rats and four animals for the control rats.

## Conclusions

- HPLC analysis of multiple proteins in bronchoalveolar lavage fluids provides a powerful, new approach for quantifying the translocation of blood compartment constituents into the lung's airspaces, as observed in permeability pulmonary edema.

- Qualitative and quantitative differences in the profiles of the various blood proteins detected by the HPLC method following lung injury may serve to provide fundamental information on the underlying nature of the injury; e.g., relative conductances of the different proteins and "effective" air-blood barrier pore sizes.

- The sensitive detection and quantification of lavage fluid protein components that are not of blood origin, using our HPLC method, may ultimately yield a relatively noninvasive assessment of the status of the lung's cellular populations and/or extracellular matrix during pulmonary disorders.

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