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Mucus Glycoprotein Secretion by Tracheal Explants: Effects of Pollutants

by Jerold A. Last* and Tokio Kaizu*

Tracheal slices incubated with radioactive precursors in tissue culture medium secrete labeled mucus glycoproteins into the culture medium. We have used an in vivtro approach, a combined method utilizing exposure to pneumotoxins *in vivo* coupled with quantitation of mucus secretion rates *in vitro*, to study the effects of inhaled pollutants on mucus biosynthesis by rat airways. In addition, we have purified the mucus glycoproteins secreted by rat tracheal explants in order to determine putative structural changes that might be the basis for the observed augmented secretion rates after exposure of rats to H_2SO_4 aerosols in combination with high ambient levels of ozone.

After digestion with papain, mucus glycoproteins secreted by tracheal explants may be separated into five fractions by ion-exchange chromatography, with recovery in high yield, on columns of DEAE-cellulose. Each of these five fractions, one neutral and four acidic, migrates as a single unique spot upon cellulose acetate electrophoresis at pH values of 8.6 and 1.2. The neutral fraction, which is labeled with [^{3}H] glucosamine, does not contain radioactivity when $Na_2\,^{35}SO_4$ is used as the precursor. Acidic fractions I-IV are all labeled with either ^{3}H-glucosamine or $Na_2\,^{35}SO_4$ as precursor. Acidic fraction II contains sialic acid as the terminal sugar on its oligosaccharide side chains, based upon its chromatographic behavior on columns of wheat-germ agglutinin-Agarose. Treatment of this fraction with neuraminidase shifts its elution position in the gradient to a lower salt concentration, coincident with acidic fraction I. After removal of terminal sialic acid residues with either neuraminidase or low pH treatment, the resultant terminal sugar on the oligosaccharide side chains is fucose. These results are identical with those observed with mucus glycoproteins secreted by cultured human tracheal explants and purified by these same techniques.

We have been using the tracheal explant system, developed several years ago in the laboratories of Reid (*1*) and others (*2-7*) as a quantitative assay for monitoring effects of air pollutants and other pneumotoxins on the respiratory epithelium. We have used what we term an "in vivtro" approach, as indicated schematically in Figure 1, featuring exposure of animals (usually rats) *in vivo* to known amounts of pneumotoxin, followed by removal of their tracheae for assay of effects *in vitro*. With small animals such as rats, we routinely incubate the tracheae in tissue culture medium containing radioactively labelled precursors. The culture medium is then removed (Fig. 1) and an aliquot is treated with 5% trichloroacetic acid to precipitate the proteins present. With a suitable choice of labeled precursor and incubation time for the rat tracheal explants, essentially all of the acid-precipitable radioactivity in the medium is mucus glycoprotein, as we will dem-

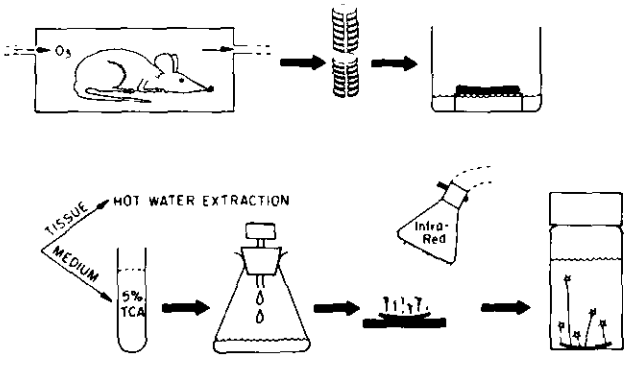

FIGURE 1. Schematic diagram of a typical experiment. Rats are exposed to a pneumotoxin (in this case, ozone) *in vivo*. Tracheae are then excised and their secretion rates are determined *in vitro* in tissue culture medium containing suitable labelled precursor(s). After incubation, an aliquot of medium is precipitated with trichloroacetic acid and filtered. The filters are dried and counted by liquid scintillation (*15*).

*California Primate Research Center, University of California, Davis, California 95616.

onstrate. What are the characteristics of such an assay? Figure 2 shows the time course of incorporation of ^{3}H -glucosamine by tracheal explants in such an assay system. The appearance of acid-precipitable glycoproteins in the culture medium can first be observed after about 3 hr. The incorporation rate is linear between about 8 hr of incubation and at least 50 hr, the maximum duration of incubation, as shown in Figure 2. In some experiments, the time course has remained linear for as long as 96 hr (data not shown). For routine assay purposes we generally incubate tracheal explants for about 24 hr. This is a convenient duration of incubation in that enough radioactivity is incorporated to enable accurate quantitation, and the incorporation is clearly first order with respect to time, so that an actual rate of incorporation is being measured.

How can we apply this system to air pollution toxicology? Figure 3 illustrates the results of an experiment in which rats were exposed to 0.8 ppm of ozone continuously for periods ranging from 1 to 90 days of exposure, and their response was evaluated by the tracheal explant assay. There is an initial decrease in the rate of glycoprotein secretion by tracheal slices as compared to the rate of secretion by slices prepared from matched control rats. This decrease lasts for 2 to 3 days, as evaluated by statistical significance; the secretion rate once again returns to the control values after a period of about 5 to

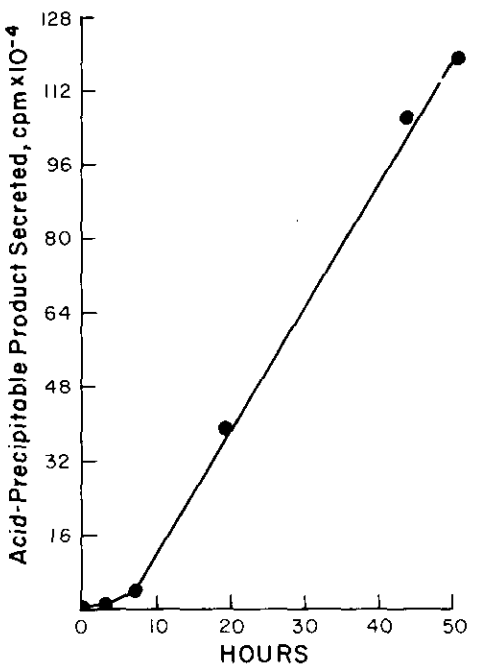

FIGURE 2. Time course of incorporation of ^{3}H-glucosamine by rat tracheal explants into acid-precipitable glycoproteins released into the culture medium.

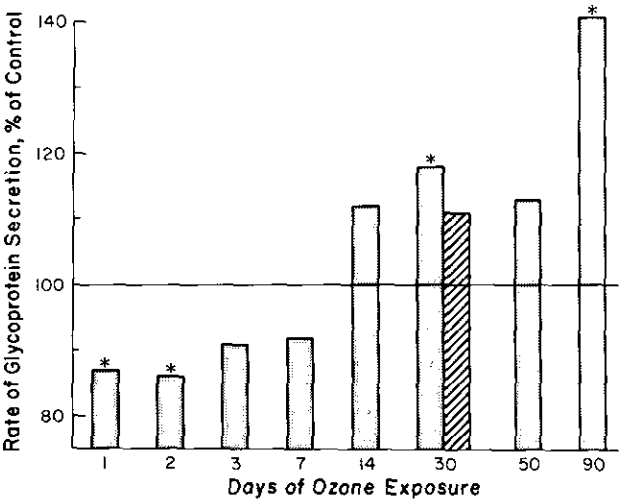

FIGURE 3. Glycoprotein secretion rates (expressed as percent of control value = 100%) of tracheal explants prepared from intact rats exposed to 0.8 ppm of ozone for the lengths of time indicated on the abscissa (*15*). The hatched bar at 30 days represents results from a separate experiment to indicate the reproducibility of the results obtained by this technique.

10 days (in different experiments). If the rats are exposed to ozone for periods longer than about a week, the secretion rate of their tracheal slices becomes greater than that of the controls. The secretion rate seems to increase continuously with longer durations of exposure, up to 90 days in Figure 3, and up to 180 days of exposure in other experiments we have performed. The hatched bar at 30 days shows the results of a separate exposure performed some 6 months after the one that had given rise to the rest of the data presented in Figure 3.

The tracheal explant system is sensitive not only to the duration of exposure of rats to ozone, but also to the concentration of ozone to which the rats are exposed. The dose-response characteristics of this system to ozone after 3 days are shown in Table 1. A statistically significant decrease in secretion rate is observed with 0.8 ppm of ozone (see Fig. 3) and with 0.6 ppm of ozone (Table 1). An insignificant decrease is observed at 0.4 ppm and no effect is seen at 0.2 ppm (Table 1). The data of Table 1 also illustrate one of the difficulties in working with the tracheal explant system. Comparisons between matched controls and experimental rats are very precise within a given experiment. In a single experiment the size of tissue slices and the matching of rats can be controlled rigorously. A single batch of medium can be used so there are minimal errors introduced by pipetting and addition of labeled precursor to the medium. On the other hand, comparisons between separate experiments are much more difficult, as shown in Table 1. Small differences in technique

from day to day result in variations in the absolute value for control rats such that data within a chronic exposure regimen must usually be normalized to control values; hence, the expression of the data in Figure 3 as percent of control. In addition, growth of the rats during a given chronic exposure experiment is appreciable and can also affect these results.

The tracheal explant system can be used with animals other than rats. We have demonstrated elsewhere (*8*) that tracheal explants from a wide variety of experimental animals, as well as human tracheal and/or bronchial explants, can be used in this system. In Table 2 we present a comparison of tracheal explant secretion rates for multiple tracheal slices prepared from individual bonnet monkeys exposed to either 0, 0.5, or 0.8 ppm of ozone for 7 days. While the increased secretion rate after 7 days suggests that there may be a species difference between monkeys and rats, we obviously need to look at many more monkeys to confirm these results. Nonetheless, clearly the technique is applicable to animals other than rats, especially nonhuman primates.

We can also use this system to study effects of pollutants other than ozone. For example, we have studied effects of exposure of rats to sulfuric acid aerosols, alone and in combination with ozone (*9*). Some representative results we have observed in this assay with sulfuric acid aerosols and/or ozone are presented in Table 3. While 0.5 ppm of ozone by itself causes a decrease in the observed secretion rate after three days, the combination of sulfuric acid aerosol with ozone causes a significant increase in this measured value. Similarly, these agents potentiate one another after 14 days of continuous exposure (Table 3). Sulfuric acid aerosol, by itself, is without affect on matched rats, as measured by their tracheal explant secretion rate, at these levels tested (*9*). Thus, exposure of rats to the combination of ozone and sulfuric acid aerosol (0.5 ppm of ozone; 0.01-5 mg/m^3 of sulfuric acid aerosol) (*9*) causes changes in the tracheal explant secretion rate that are not only quantitative, but are qualitative over the 2-3 day exposure interval. We are presently quite vigorously studying the biochemical and/or cellular basis of such pollutant-induced changes in secretion rates.

Table 2. Tracheal explant secretion rates for tracheal slices from monkeys exposed to ozone.

O_3 level, ppm	Secreted precipitable labeled molecules after 7 days of O_3 exposure
Control	12,362 ± 2,100
0.5	16,679 ± 3,767
0.8	21,949 ± 6,383

Table 3. Rate of secretion of mucus glycoproteins by tracheae from rats exposed to H_2SO_4-O_3 mixtures.

Time exposed *in vivo*, days	H_2SO_4, mg/m^3	O_3, ppm	Rate of secretion, % of control value[a]	*p* value
3	1.1	0.5	127	<0.005
14	1.1	0.5	132	<0.001
3	1.1	None	100	NS[b]
3	None	0.5	84	NS
14	None	0.8	112	NS

[a]Tracheal explants were incubated 22-24 hr; in all cases, $n = 14$ slices.
[b]Not significant.

Once we had documented quantitative changes in mucus secretion rates by tracheal explants from rats exposed to various pollutants, we turned our attention to the question of whether there was also a qualitative change in the mixture of glycoproteins being secreted by the explants. In order to separate the mucus glycoproteins into various fractions on ion-exchange columns, it was first necessary to solubilize these compounds. In the absence of prior treatment to solubilize the glycoproteins, yields from ion-exchange columns were very low and erratic. Other workers have used reduction and alkylation of sulfyhydryl groups in the protein backbone involved in glycoprotein crosslinking for this purpose (*10, 11*); however, since we were most interested in determining changes in the carbohydrate side chains rather than the protein backbone, we chose limited digestion with papain as our initial solubilization

Table 1. Effect of exposure of rats to ozone on the rate of glycoprotein secretion by their tracheal explants.

Ozone concentration, ppm[a]	Secretion rate (mean ± SD), cpm		Total *n*	Exposed control, %	*p* value
	Control	Exposed			
0.6	19,110 ± 2,230	16,972 ± 2,269	24	89	<0.02
0.4	14,913 ± 2,309	14,327 ± 2,118	24	96	NS[b]
0.2	16,870 ± 2,770	17,034 ± 2,503	22	101	NS

[a]Exposures were for 8 hr/day for 3 days to the indicated concentration of ozone.
[b]Not significant.

step, as shown schematically in Figure 4. After papain digestion we can chromatograph the resultant mixture of glycoproteins on DEAE-cellulose and recover the applied radioactivity essentially quantitatively. As shown in Figure 5, an idealized version of such a column, the ^{3}H-glucosamine-labeled glycoproteins separate into one neutral and two acidic glycoprotein fractions under the conditions we initially used for this purpose (a linear gradient from 0 to 1 *M* lithium chloride in 50*mM* sodium acetate-acetic acid buffer at pH 4.4). We observe three peaks (A, B, and C) that are eluted at the same positions from the DEAE-cellulose column whether we use ^{3}H-glucosamine or ^{14}C-threonine as the precursor for the glycoproteins. When ^{35}S-sulfate is used as the labeled precursor, most (if not all) of the label appears in the most acidic fraction from the DEAE-cellulose column, the fraction that we call peak C. This material is chromatographically indistinguishable from peak C labeled with ^{3}H-glucosamine or with ^{14}C-threonine. Both of these two acidic peaks are eluted from the column at positions distinctly different from those of hyaluronic acid or chondroitin sulfate treated in an identical manner, added as internal standards and assayed chemically by determination of their constituent uronic acid components. If a mixture of the two acidic fractions is incubated at 37°C in an appropriate buffer overnight for 18-24 hr, then rechromatographed, their elution positions are unchanged from the original chromatographic mobilities observed. If the same mixture of fractions B and C are incubated in buffer containing neuraminidase from *Vibrio cholerae*, an enzyme that

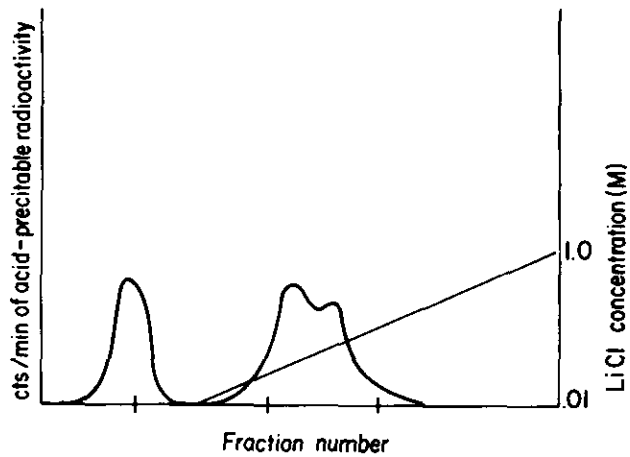

FIGURE 5. Idealized representation of the elution of labelled glycoproteins, treated as in Figure 4, from a column of DEAE-cellulose.

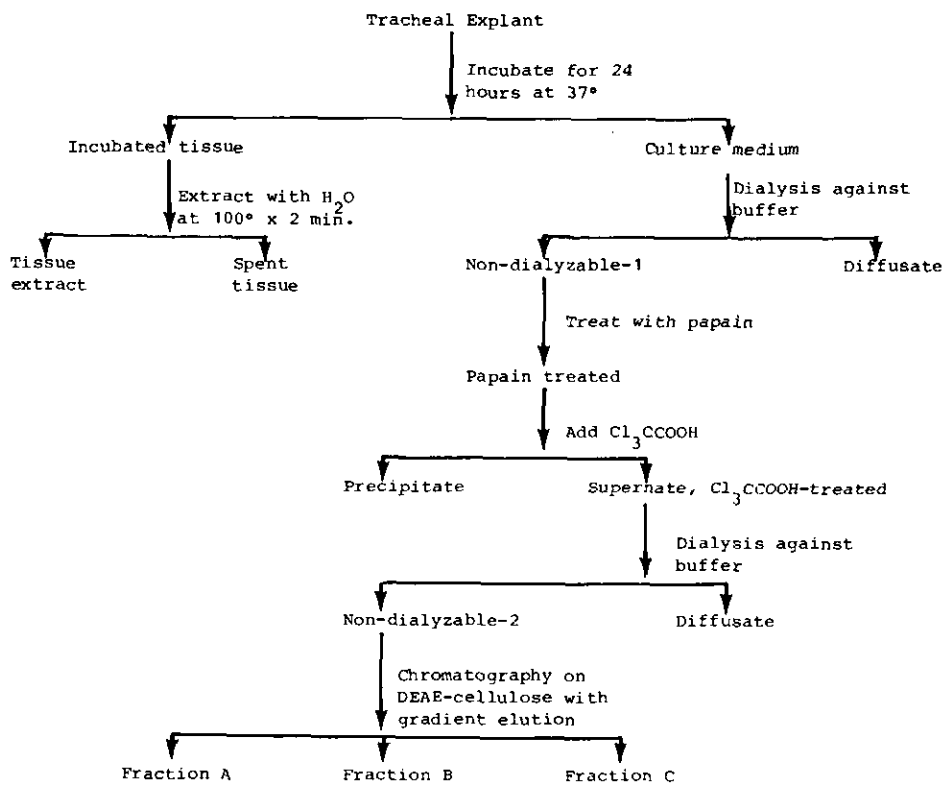

FIGURE 4. Diagrammatic representation of the techniques used to purify mucus glycoproteins from the culture medium of tracheal explants.

specifically cleaves sialic acid residues from the nonreducing end of oligosaccharide chains, we then observe only one peak after incubation; this single peak is chromatographically indistinguishable from the less acidic peak, peak B of Figure 5. Thus, we can conclude that the more acidic peak (peak C) from the DEAE-cellulose column contains glycoproteins containing oligosaccharide side chains with terminal sialic acid residues in their structure. The sulfur-35 labeling experiment suggests that, in addition to its sialic acid content, the more acidic fraction is also more heavily sulfated than is fraction B.

In Figure 6 we indicate schematically the structure of the tracheobronchial mucus glycoproteins that we are studying in such an experiment. They are long extended proteins in a backbone configuration with short oligosaccharide side chains coming off, perhaps as frequently as every fifth or sixth amino acid residue of the protein. The protein backbones are high in serine and threonine, to the extent of about 40-50% of their total amino acid content, and the oligosaccharide side chains occur attached to serine or threonine residues in ester linkages through the hydroxyl groups of the serine or threonine residues. The oligosaccharide side chains contain *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, fucose, and, when present, sialic acids. These are presumably the only sugars present. The length of the side chains is a subject of some controversy, but they are usually assumed to be approximately six or eight residues long (*12*). We have evidence, based on cleavage of the serine or threonine linkages to the oligosaccharides under alkaline conditions, that the oligosaccharide side chains that we are studying are relatively homogeneous in size, as indicated by their mobility on columns of Sephadex G-50 (data not shown).

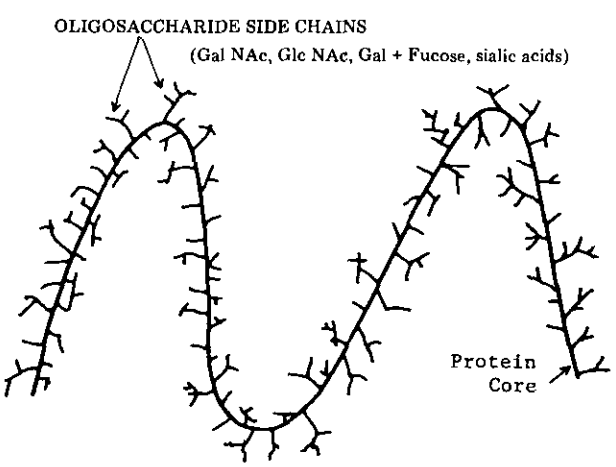

FIGURE 6. Idealized structure of tracheobronchial mucus glycoproteins.

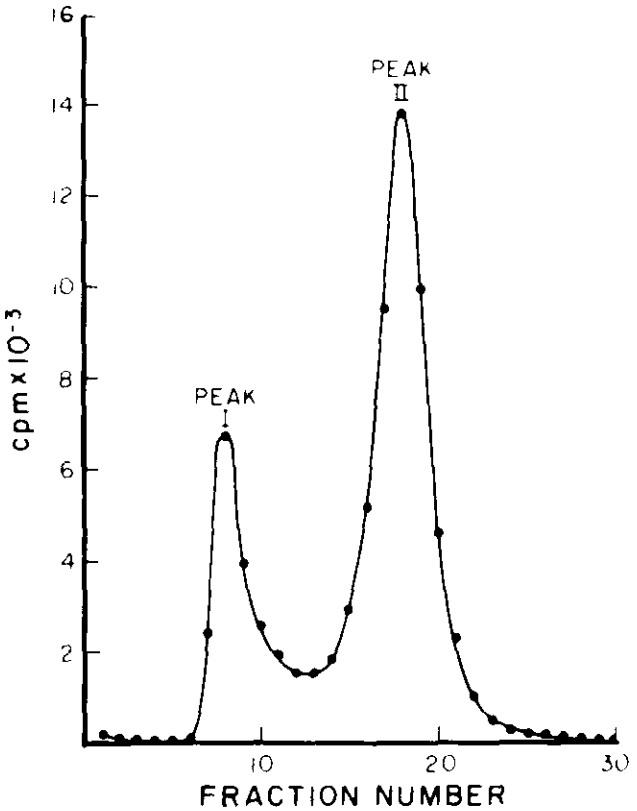

FIGURE 7. Elution of ^{3}H-glucosamine-labelled glycoproteins, prepared from exhaustively dialyzed tracheal culture medium, from a column of BioGel A150m (*15*).

One technique that has been used extensively for the characterization of mucus glycoproteins secreted by cultured tracheal explants has been fractionation upon sizing columns, usually either agarose or polyacrylamide (*4, 5, 13, 14*). Under appropriate conditions of chromatography, two peaks of mucus glycoproteins are observed to be eluted from such sizing columns. The first peak, which elutes at approximately the void volume of the column, is extremely large and presumably represents aggregated glycoproteins. The second peak, which is included in the column, may also be shown to be a glycoprotein fraction (*5*).

The ratio of glycoproteins in the two peaks has been used by others (*4*), as well as by ourselves (*15*), as a probe for observing changes in the structure of glycoproteins being secreted by tracheal explants. We have published (*15*) evidence that chronic exposure of rats to ozone causes changes in the ratio of the two peaks from a BioGel A150m column, such that the lower molecular weight included peak is augmented at the expense of the first peak, as shown in Figure 7. In simpler terms, there was a shift in the relative number of counts from peak I to peak II upon

exposure of the rats to ozone in those experiments. We therefore turned our attention to the question of whether the peaks from the BioGel A150m column corresponded in any way to the fractions we could isolate by chromatography on DEAE-cellulose using the techniques illustrated schematically in Figure 4. However, this experimental design required that we omit the papain digestion step. Thus, the isolated fractions from the BioGel columns were applied directly to DEAE-cellulose and eluted under the gradient conditions illustrated in Figures 4 and 5. Since we anticipated low yields by this procedure, we labeled the glycoproteins with sulfate rather than with ^{3}H-glucosamine to enhance the counting efficiency. Under these conditions, we observed a third fraction eluted from the BioGel column, as a shoulder on the high-molecular weight side of peak II, as shown in Figure 8. Thus BioGel chromatography of ^{35}S-labeled material gave us three fractions, more or less well resolved from each other, which we labeled A, B, and C as shown in Figure 8. To make a long story short, each of the three fractions, A, B, and C, corresponded to one of the peaks from the DEAE-cellulose column, with only minor cross-contamination of the fractions with one another. Thus fraction A, the high molecular weight peak I, corresponded to the neutral glycoprotein fraction from the DEAE-cellulose columns. Similarly fraction C, the included peak II, corresponded to the first acidic glycoprotein fraction B from DEAE-cellulose. Finally fraction B, the high molecular weight shoulder on peak II, corresponded to fraction C, the most acidic glycoprotein fraction from DEAE-cellulose. Thus, we can express the results of our earlier experiments, in which we had shown a shift in the ratio of the glycoproteins from peak I to peak II after exposure of rats to ozone, in more biochemical terms. The chronic exposure of rats to ozone apparently induces a shift in the composition of the mucus glycoproteins being secreted by their tracheal explants such that the product is relatively more acidic than is the mixture of mucus glycoproteins from control rat explants. This result is not unexpected (*1, 9*) considering the results of others who have exposed rats to various irritant stimulants under chronic conditions.

We have recently improved our DEAE-cellulose chromatographic techniques by changing to a less steep lithium chloride gradient. Under these conditions one neutral fraction is still eluted, but four acidic fractions are now eluted rather than two. Each of these four acidic fractions is different than the other as evaluated by cellulose acetate electrophoresis or by rechromatography on DEAE-cellulose. We are currently using immobilized lectin columns as a technique for probing the structure of the sugars on the oligosaccharide side chains within the components of the various fractions. We have been able to document shifts in the ratios of these chromatographic peaks relative to one another upon exposure to ozone in this new system. For example, as shown earlier (Fig. 3, Tables 1 and 3), short-term acute exposure to ozone at 0.5 ppm for three days results in a significant decrease in the mucus secretion rate by the tracheal slices. When the papain-treated glycoproteins from control and exposed rat tracheal explants are chromatographed on DEAE-cellulose, we find a shift to a mixture that is relatively deficient in acidic mucus glycoproteins from the tracheae of ozone-exposed rats. Experiments are currently in progress to try to elucidate which acidic fraction is being decreased under these circumstances; preliminary data implicate the sialic acid-containing fraction as being present in relatively smaller amounts.

In conclusion, we have developed the tracheal explant system as a technique for quantitating the

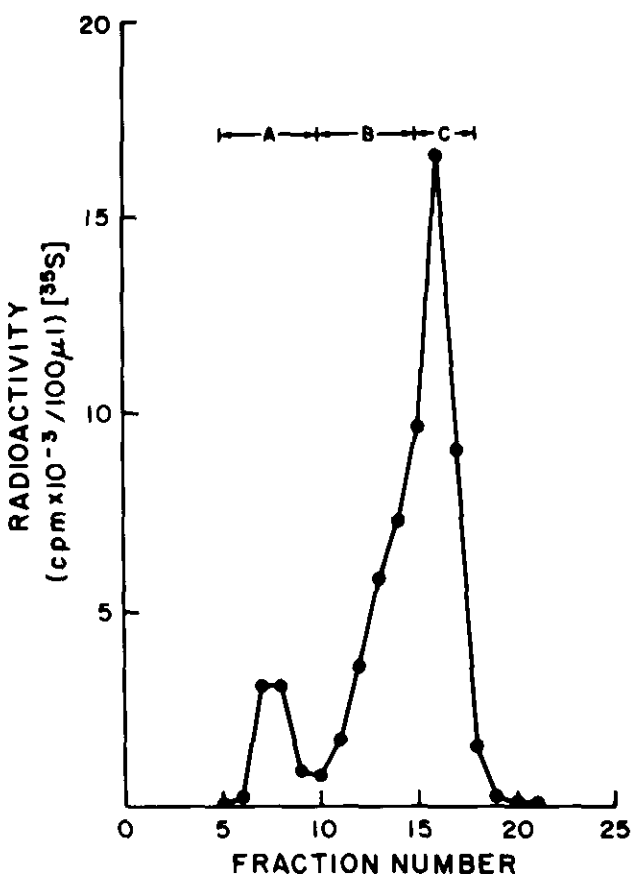

FIGURE 8. Elution of ^{35}S-labeled glycoproteins, prepared as in Figure 4, from a BioGel A150m column.

effects of exposure to air pollutants and other pneumotoxins on the respiratory airways of small laboratory animals, and on non-human primates. These techniques are relatively easy to perform and give reproducible quantitative results. Thus, any airborne noxious agent can be evaluated with this system. We are currently concentrating our attention upon trying to elucidate the molecular basis for changes in secretion rates both in terms of decreased secretion rates in short-term acute exposures to ozone and increased secretion rates in longer-term chronic exposure regimens to various pollutants. These techniques promise to have a great deal of value both for mission-oriented research (screening of airborne pollutants, gasses, and chemicals for effects on the respiratory airways) and for basic research studies (elucidation of the structure of tracheobronchial mucus in health and disease).

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