Thorax (1975), **30**, 382.

The composition of massive lesions in coal miners

J. C. WAGNER[1], F. S. WUSTEMAN[2], J. H. EDWARDS[1], and R. J. HILL[1]

Medical Research Council, Pneumoconiosis Unit, Llandough Hospital, Penarth, Wales[1] *and Biochemistry Department, University College, Cardiff, Wales*[2]

Wagner, J. C., Wusteman, F. S., Edwards, J. H., and Hill, R. J. (1975). *Thorax*, **30**, 382–388. **The composition of massive lesions in coal miners.** The nature of the material forming the massive lesions in the lungs of coal workers has never been demonstrated. The concept that it was in fact massive fibrosis, implying that it consisted of collagen impregnated with coal dust, has been challenged only during the last ten years. It was agreed that the best chance of obtaining more definite information was from a combined study of the biochemical, pathological, ultrastructural, and immunological features of a number of lungs containing these lesions. Six cases which were found to contain suitable material were studied. The preliminary results obtained suggest that collagen is present in the capsule of these lesions but that at the centre it is replaced by another insoluble protein or proteins which is probably stabilized by some form of cross-linking. This protein complex accounts for one-third of the weight of the lesions, the remaining two-thirds consisting of approximately equal amounts of mineral dusts and calcium phosphate. Serum proteins were also observed but their association with the lesions has yet to be determined.

Until ten years ago it was generally accepted that the massive lesions occurring in cases of complicated coal workers' pneumoconiosis were masses of fibrous tissue in which collagen fibres enmeshed foci of coal dust, hence the term progressive massive fibrosis (PMF). This view was first challenged by Nagelschmidt and his co-workers in 1963. They showed that if the hydroxyproline content of the lesions could be taken as an indication of the amount of collagen present, there was no significant increase in the collagen of the so-called PMF lesions compared with the surrounding involved lung tissue.

Following this study, a series of investigations was undertaken by the Medical Research Council Pneumoconiosis Unit and the Biochemistry Department of the University College, Cardiff to establish the nature of these massive lesions, combining biochemical, immunological, and pathological studies. Preliminary findings have been published elsewhere (Jones, Edwards, and Wagner, 1972; Wagner, 1972; Wusteman, Gold, and Wagner, 1972). In this paper we illustrate our views on the subject and indicate the direction of our future work.

MATERIAL

The results of earlier analyses were difficult to correlate since the samples used were not obtained from the same lesions, and many specimens were taken from necrotic lesions or from cases in which post-mortem autolysis was severe. We therefore developed a system in which only lungs removed surgically, or cases in which the necropsy had taken place within 12 hours of death, were acceptable. (The histological, electron microscopic, biochemical, and immunological studies were all undertaken on tissue from the same area.) There is a further delay because the presence of *Mycobacterium tuberculosis* must be ruled out before the biochemists macerate the tissue. In every case, the tissue was kept in a deep freeze at −20°C until both the cultural and animal inoculation tests were reported as negative. Then the tissue was thawed and the precise fragments for further study were selected by the pathologist and biochemist. Tissues from a total of six patients with PMF were suitable, one from the Pathology Department of the Welsh National School of Medicine and the rest from the Medical Research Council's Pneumoconiosis Unit, Penarth. The

cases finally selected for study (out of a total of 10 examined) had lesions that were extensive enough for analysis of one or more grossly homogeneous portions of approximately 1 g dry weight. Material was selected from different parts of the lesion and from unaffected normal areas of the same and of control lungs from patients of the same sex and age range (Fig. 1).

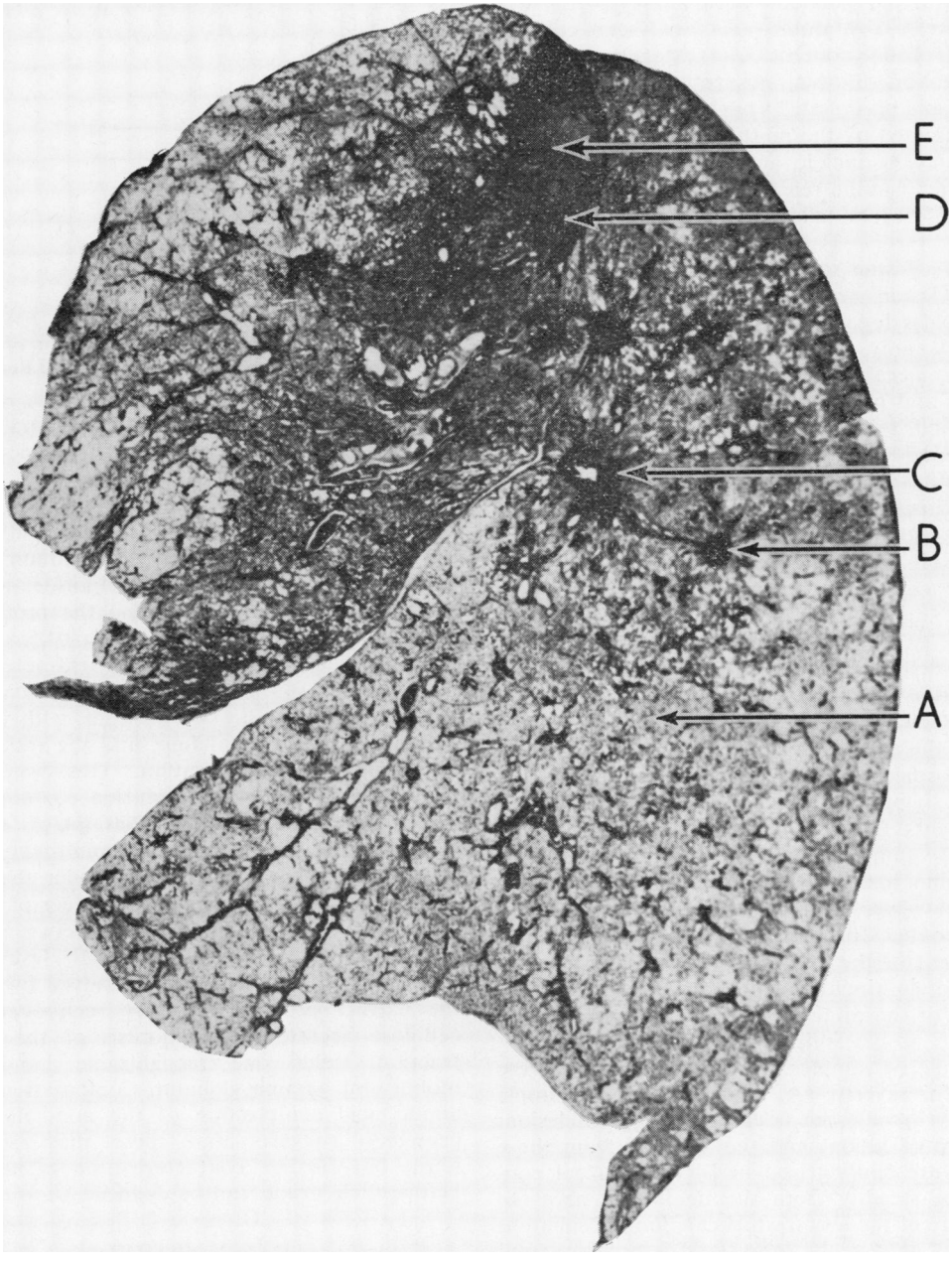

FIG. 1. *Whole lung section showing the areas selected for examination: A—control tissue; B—simple nodule; C—coalescent nodule; D—edge of lesion; E—centre of lesion.*

BIOCHEMISTRY

METHODS Deep-frozen necropsy material was immersed in ethanol, washed with acetone and ether, and then dried in air. Tissue samples were hydrolysed in 6 M hydrochloric acid for 24 hours at 110°C, and portions were analysed for total amino acids (by amino acid analyser), collagen (hydroxyproline content ×7·4) (Stegemann, 1958), calcium (by atomic absorption spectrophotometry), and phosphate (Vogel, 1961). Coal content was estimated by the method of Bergman (1966), and deoxyribonucleic acid (DNA) by means of the diphenylamine reaction on extracts soluble in 5% trichloracetic acid at 90°C (Schneider, 1957).

RESULTS Tissue samples from the centre of the massive lesion were found to contain as much as 47% coal (dry weight). Unexpectedly, the dry solid was grey, and further analysis detected a second mineral component which contained both calcium and phosphate. In different samples this was found to be present (allowing for normal background levels) in a constant ratio of 1·8 atoms of calcium for every phosphate (Wusteman *et al.*, 1972). This form of calcium phosphate was finely divided and, though not uniformly distributed throughout the lesion, was not present in massive palpable aggregates. Calcium phosphate often contributed significantly to the bulk of the lesion (up to 30% by weight), and detectable increases in tissue calcium (twice that of the control area) were found in apparently normal areas adjacent to the PMF lesion.

Since the assays for amino acid and coal had to be performed on separate samples, it was not possible to prepare an accurate balance-sheet of components for one homogeneous area of the lesion. By summing the coal, calcium phosphate, and amino acid residue contents for the same region, however, 77–100% of the dry weight could be accounted for. Thus the only other major component of the lesion is protein. The range of total protein (including collagen, which is itself 27±3% of the total protein) in the central lesion is 28–67% of dry weight (average 41%).

A range of samples from the same lung were graded for severity (A, control tissue; B, simple nodules; C, coalesced nodules; D, edge of lesion; E, centre of lesion) and the analyses from these samples are summarized in Figure 2. Coal content rises steadily from the unaffected areas to the centre of the lesion while calcium phosphate is abundant only in the lesion and is not increased significantly in the 'simple' or 'coalesced' nodules. While DNA content drops in the more severely

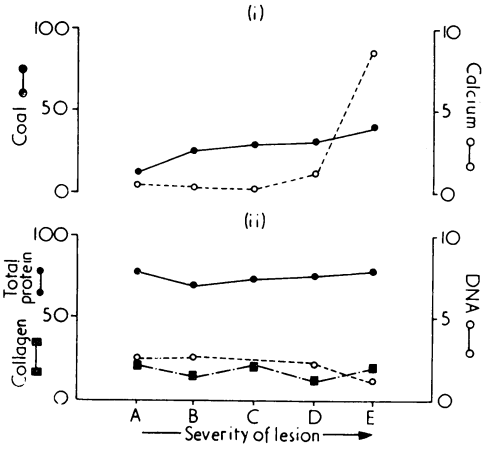

FIG. 2. *Composition of tissue from PMF lung (i) expressed as percent total dry weight of sample and (ii) expressed as percent non-mineral dry weight.*

affected areas the protein content rises slightly and collagen fluctuates but does not decrease or increase in a consistent fashion.

The protein(s) accompanying the collagen are not readily characterized. Of a range of extractants tested (including 2% aqueous sodium dodecyl sulphate and 0·2 M tris hydrochloride, pH 8·3) the most effective in dissolving the protein was found to be 8 M urea containing 0·05% dithiothreitol. The solid was first extracted with 400 ml/g of 0·05 M disodium ethylenediaminetetracetate (EDTA) (in two portions, with vigorous stirring) to remove calcium, and the residue was recovered by centrifugation. This was stirred vigorously with the urea solution containing the sulphydryl reducing agent (200 ml/g), and the soluble extracts were separated from the inextractable residue by centrifugation. Under these conditions 5% of the amino acid residues of control tissue were rendered soluble while between 13 and 40% of the material of the PMF lesion were extracted. When the urea is removed by dialysis the extract protein is mostly reciprocated but cellulose acetate electrophoresis of the soluble fraction yields one recognizable band, with mobility of gamma globulin. Computation of the amino acid composition of the non-collagenous protein(s) suggests that these most resemble fibrin but further characterization is necessary before their origin can be deduced.

ELECTRON MICROSCOPY

METHOD Tissues from postmortem examination were fixed as soon as possible after death in 2·5%

phosphate buffered glutaraldehyde at 4°C using 2 mm slices. After initial fixation, tissues were further cut into pieces 2×1 mm, washed in buffer, post fixed in 1% esmium tetroxide dehydrated in graded alcohols, and embedded in epoxy resin (Durcupan ACM Fluka). Sections were cut on a LKB Ultratome III using glass knives, initially at 1 μm, stained with toluidine blue for light microscopy and finally at 60–100 nm for electron microscopy. They were stained with uranyl acetate and lead citrate and examined in an AEI EM6B microscope at an accelerating voltage of 60 kV.

RESULTS In the small fibrotic nodules, the lesions consist of dense masses of collagen fibres with occasional fibroblasts and fragments of coal dust, some of which are within phagocytes. In the large stellate nodules the periphery of the lesion is collagen but toward the centre the pattern of the fibres becomes interrupted by an amorphous material that appears to spread between the collagen fibres and separates them. The amorphous material becomes more and more predominant, and sections from the middle of the nodules show only a few scattered bundles of collagen lying in an acellular matrix. These changes are even more marked in sections from the massive lesions; these have dense collagen at the edge (Fig. 3) but numerous sections from the centre (from the firm non-necrotic tissue) consist of this amorphous material surrounding fragments of coal dust (Figs 4–7). These findings would tend to confirm the biochemical studies.

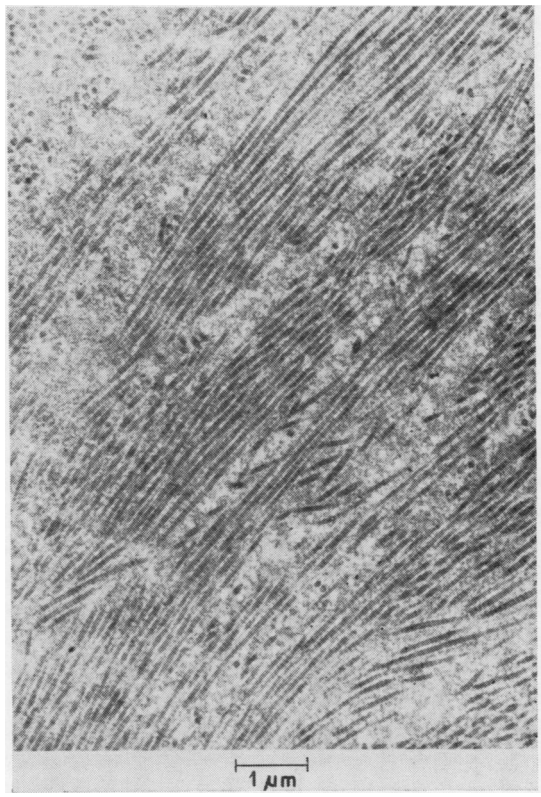

FIG. 3. *Electron micrograph of area D showing regular bundles of collagen fibres.*

IMMUNOLOGICAL ANALYSIS

Previous work on the absorptive properties of coal dust suggested that coal in PMF lesions might hold protein absorbed to the dust particles and that these proteins could contribute to the overall structure of the lesion. Also, the association of rheumatoid factor with PMF cases, its antibody activity with respect to altered IgG, and the direct presence of these immunoglobulins in lesions of rheumatoid lung disease (De Horatius, Abruzzo, and Williams, 1972) suggested that PMF lesions could be investigated for immune complex deposition.

On a quantitative basis, the presence of non-bound (i.e., 'elutable') materials was determined with specific antisera while immune complexes were first dissociated and individual components determined after separation. The results presented are those of a pilot study of methods for the analysis of solid lesions immunologically.

METHOD (1) PMF material from the centre of the lesion (0·5 g) was extracted with 25 ml 0·1 M EDTA for 16 h at 4°C and centrifuged and the supernatant and sediment were used for analysis.

Supernatant The supernatant was dialysed against 0·1% NaCl and concentrated ×10 by air dialysis and then tested by double gel diffusion against anti-human serum, fibrinogen, IgA, IgG, and IgM. The results obtained were as follows:

Antiserum to:	
Human serum	++
Fibrinogen	+
IgA	−
IgG	++
IgM	−

Sediment The sediment was extracted with 25 ml 0·01 M NaOH at +4°C for 16 h, then dialysed against 0·01 M NaOH for 24 h at +4°C, centrifuged, and concentrated ×10. The suspension was filtered to remove particles greater than

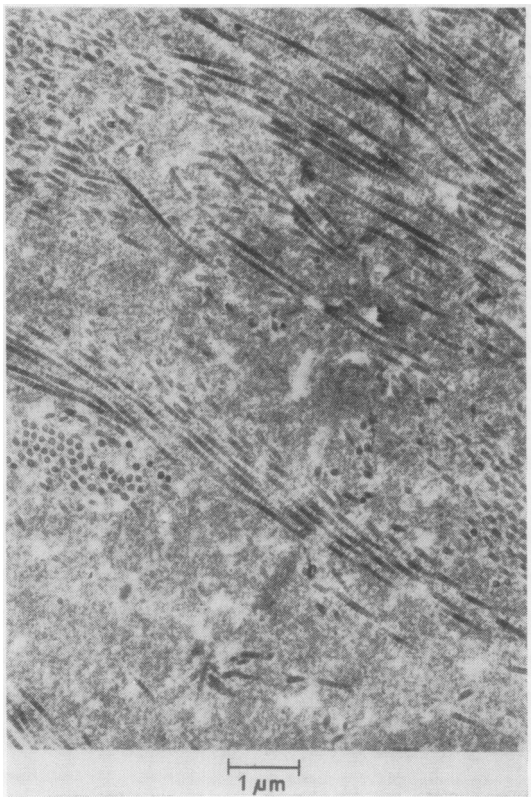

FIG. 4. *Electron micrograph of area C showing less collagen and large amounts of ground substance.*

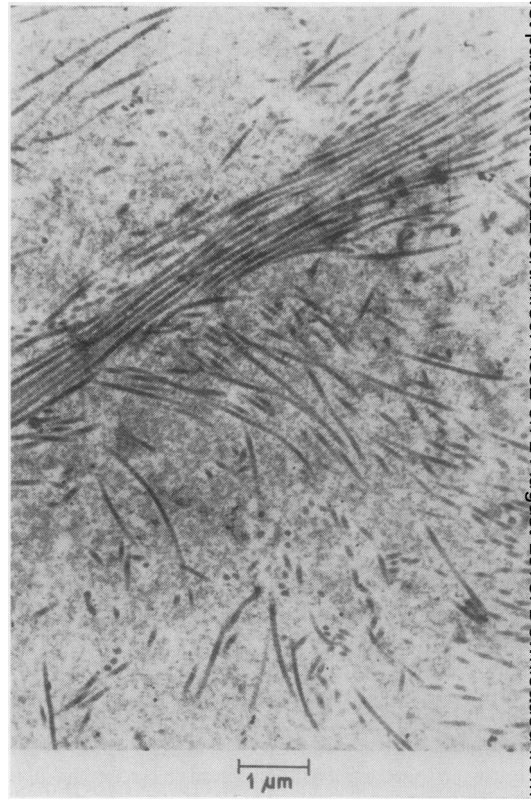

FIG. 5. *Electron micrograph of area E showing little collagen.*

3 μ and placed on a column of Sephadex G200 (90×1·5 cm) equilibrated with 0·01 M NaOH. The optical density of eluting protein was monitored at 280 mμ. Two peaks were observed and fractions corresponding to these (approx. 50 ml each) were pooled and dialysed against 0·01% NaCl (5 changes) and concentrated to 0·5 ml by air dialysis, then tested by gel diffusion against specific antisera. The following results were obtained:

	Peak 1 high M wt	Peak 2 lower M wt
Anti-human serum	+	+
Fibrinogen	+	−
IgA	+	−
IgG	+	+
IgM	−	−

(2) Another approach was to immunize animals with extracts of PMF and let the animal 'decide' which proteins were present.

A PMF lesion (approx. 5 g) was subjected to Hughes Press extraction, yielding 600 mg soluble material. Equal volumes of the extract at 35 mg/ml and Freund's Complete Adjuvant (Difco) were emulsified together and inoculated into two rabbits intramuscularly using a regimen of four injections at weekly intervals. The animals were bled 10 days after the last injection. The antiserum was then used to develop the PMF extract on immunoelectrophoresis. Several precipitin arcs were seen, some undoubtedly of serum protein origin. Thus the antiserum was absorbed out using:

(a) ethyl chlorformate insolubilized human plasma (Avrameas and Ternynck, 1967) at 35 mg insolubilized protein per ml of antiserum; and

(b) human plasma 1 vol+4 vol antiserum.

After incubation at 37°C for 2½ h and at 4°C for 16 h the absorbed sera were again used to develop the PMF extract. Three arcs remained after absorption. One lay in the globulin position, and the

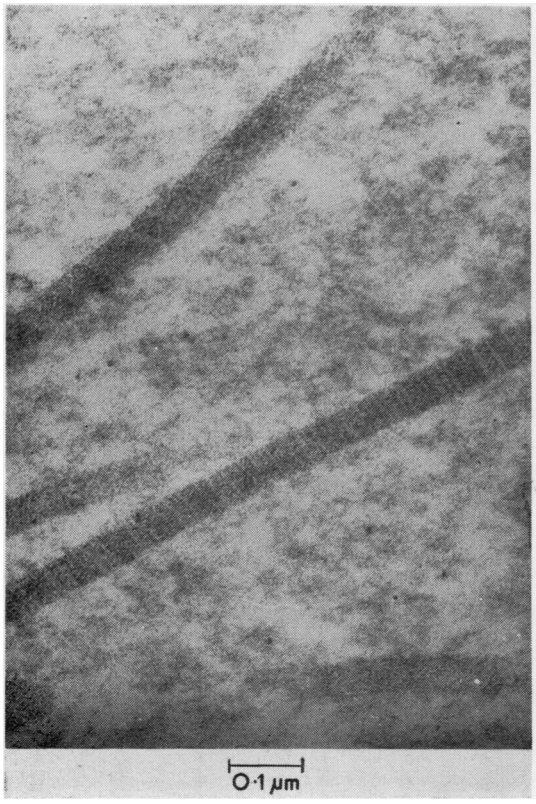

FIG. 6. *High power view of area E.*

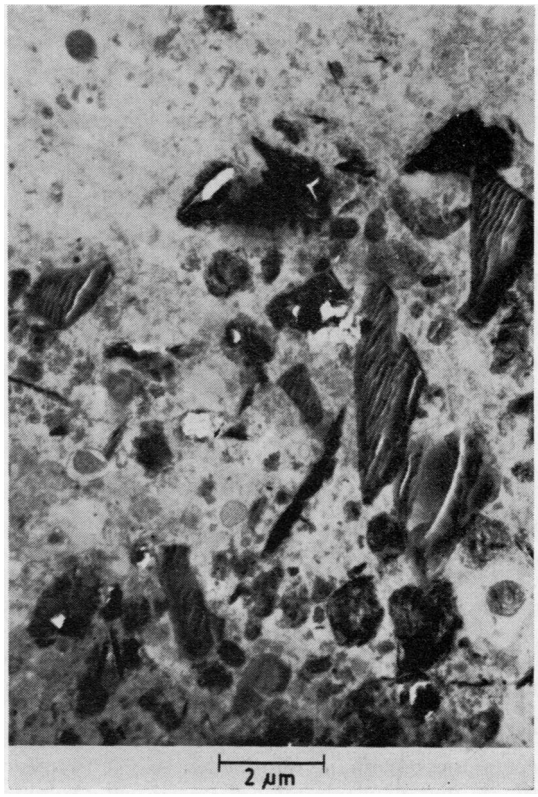

FIG. 7. *High power view of area E showing mineral dust.*

other two had β-globulin mobility. Further analysis of the materials responsible for these arcs is in hand.

DISCUSSION

Though collagen constitutes less than one-third of the protein which accumulates in the lesion of PMF, fibrosis is clearly taking place. The glycosaminoglycans (acid mucopolysaccharides) in the PMF lesion have been found to change in a way closely similar to that in other forms of fibrosis (Fig. 8) and close parallels with the changes in calcifying pleural plaques have already been reported (Wusteman *et al.*, 1972). Since pleural calcification takes place where 90% of the protein is collagen the deposition of calcium salts in the centre of the PMF lesion need not be related to the presence of the other major component, the non-collagenous protein.

Non-collagenous protein increases while the DNA (and glycosaminoglycan—Wusteman *et al.*,

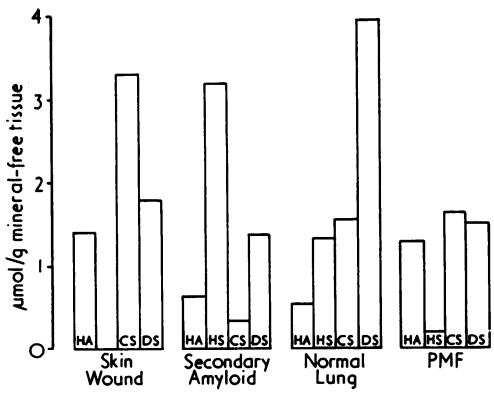

FIG. 8. *Glycosaminoglycan (mucopolysaccharide) composition of the PMF lesion: HA—hyaluronic acid (expressed as uronic acid); HS—heparan sulphate (expressed as glucosamine); CS—chondroitin sulphates (expressed as galactosamine); DS—dermatan sulphate (expressed as galactosamine).*

1972) content drops, indicating a process whereby the protein(s) are being actively deposited. Since only a small, variable proportion is soluble under conditions where globular proteins should be extracted, it seems likely that the insoluble protein(s) are stabilized by some form of cross-linking.

Our immunological studies confirm the biochemical findings of soluble and 'poorly' soluble material in PMF lesions. Serum proteins IgG, IgA, and fibrinogen were found in the soluble fraction, but whether these reflect PMF lesion material, blood from areas other than the lesion or residual vascular infiltration has yet to be determined. Certainly antisera raised by inoculating PMF into rabbits detected serum proteins and also non-serum proteins judging from the absorption study.

The dissociation experiment shows materials to be present in the lesion not extracted by simple solution. Gel filtration on Sephadex G200 columns yielded protein peaks that contained immunoglobulins. It is tempting to speculate the presence of immune complex deposition especially as IgG and IgM have been shown to be present in related rheumatoid lung disease lesions (De Horatius *et al.*, 1972); also, rheumatoid factor is present in the majority of PMF cases and this reacts immunologically with 'altered' IgG.

While we have achieved some degree of success using the techniques outlined above it is unfortunate that they rely upon material being soluble. Complete solution of the PMF lesion is not possible and this prevents an absolute quantitation of individual components by immune techniques.

Further studies will be aimed at a positive identification of the insoluble protein fractions and an explanation of the cross-linking process.

REFERENCES

Avrameas, S. and Ternynck, T. (1967). Biologically active water insoluble protein polymers. *J. biol. Chem.*, **242**, 1651–1659.

Bergman, I. (1966). Determination of coal in formalin-fixed pneumoconiotic lungs. *Analytical Chemistry*, **38**, 441.

De Horatius, R. J., Abruzzo, J. L., and Williams, R. C., Jr. (1972). Immunofluorescent and immunologic studies of rheumatoid lung. *Archives of Internal Medicine*, **129**, 441.

Jones, B. M., Edwards, J. H., and Wagner, J. C. (1972). Absorption of serum proteins by inorganic dusts. *British Journal of Industrial Medicine*, **29**, 287.

Nagelschmidt, G., Rivers, D., King, E. J., and Travella, W. (1963). Dust and collagen content of lungs of coal-workers with progressive massive fibrosis. *British Journal of Industrial Medicine*, **20**, 181.

Schneider, W. C. (1957). Determination of nucleic acids in tissues by pentose and analysis. *Methods of Enzymology*, **3**, 680.

Stegemann, H. (1958). Mikrobestimmung von Hydroxyprolin mit Chloramin-T und p-Dimethylaminobenzaldehyd. *Hoppe-Seylers Zeitschrift für physiologische Chemie*, **311**, 41.

Vogel, A. I. (1961). A Textbook of *Quantitative Inorganic Analysis*, 3rd edition, p. 810. Longmans, London.

Wagner, J. C. (1972). Etiological factors in complicated coal workers' pneumoconiosis. *Annals of the New York Academy of Sciences*, **200**, 401.

Wusteman, F. S., Gold, C., and Wagner, J. C. (1972). Glycosaminoglycans and calcification in the lesions of progressive massive fibrosis and in pleural plaques. *American Review of Respiratory Disease*, **106**, 116.

Requests for reprints to: Dr. J. C. Wagner, Medical Research Council, Pneumoconiosis Unit, Llandough Hospital, Penarth, Glamorgan.