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# Immunoquantification of Type I, III, IV and V Collagen in Small Samples of Human Lung Parenchyma

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The involvement of collagen in pathological conditions underscores the need for sensitive, collagen type-specific assays. A method for the quantification of different types of collagen in lung has been developed. Human lung parenchyma is digested with cyanogen bromide which results in almost complete solubilization of collagen to type-specific collagen peptides. The peptides are quantified using inhibition enzyme immunoassays with type-specific antibodies. The 50% inhibition value for the type I collagen assay is 1  $\mu$ g type I collagen peptides, for the type III collagen assay 350 ng type III collagen peptides, for the type IV collagen peptides, and for the type V collagen assay 50 ng type V collagen assay 50 ng type III collagen assay 50 ng type I collagen, and for the type I collagen assay 50 ng type I collagen, 26.6 ± 10.3  $\mu$ g for type III collagen, 9.6 ± 2.0  $\mu$ g for type IV collagen and 1.8 ± 0.3  $\mu$ g for type V collagen. The procedure is useful for the quantification of different types of collagen, including minor collagens, and requires only minimal sample preparation.

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Keywords: Collagen Human lung Immunoassays Quantification Solubilization

#### **INTRODUCTION**

The lung is a highly dynamic and delicate organ, which depends to a great extent on the extracellular matrix for the maintenance of its structure. A continuous network of connective tissue elements extends all the way from the airways to the alveoli and from the visceral pleura into the lung. Collagen and elastin are the two main components of the extracellular matrix. While elastin appears to be the product of a single gene, collagen is encoded for by more than 30 genes, giving rise to 19 types of collagen (Vuori and Crombrugghe, 1990; Van der Rest and Garrone, 1991). Type I, II and III collagen constitute the bulk of lung collagen, type II collagen being restricted to the cartilage of the major air vessels. Type IV collagen is present in basement membranes and determines to a large extent the tensile strength of the blood-air barrier (Konomi et al., 1981; West and Mathieucostello, 1992). Other types of collagen (e.g. type V, VI, X) are present as well, each with their own distribution, their function being unclear (Sano et al., 1981; Amenta et al., 1988; Bearman and Flint, 1986). Collagens have been related to a number of pulmonary diseases like fibrosis, Goodpasture's syndrome, and emphysema (Raghu et al., 1985; Turner et al., 1992; Cordoso et al., 1993). The involvement of collagen in pathological conditions underscores the need for sensitive, collagen type-specific assays. The most used assay to quantify collagen in lung has been by measuring hydroxyproline (Pierce and Hocott, 1960; Laros et al., 1972). This method is relatively inaccurate (some other

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proteins like elastin also contain hydroxyproline, Pierce and Hocott, 1960; Chrzanowski et al., 1980), and cannot distinguish between different types of collagen. In order to develop an accurate and precise method to quantify different types of collagen, two major demands have to be met: complete solubilization of collagen, and type-specificity. With regard to the first, lung collagen is notoriously difficult to solubilize (Clark et al., 1983). The most used technique to solubilize collagen has been limited pepsin digestion. In this way, however, only 20-50% of the collagen is solubilized and the resulting fraction is not representative for all types of collagen (Last and Reiser, 1984). Furthermore, subsequent isolation of different types of collagen is hampered by lengthy purification procedures and concomitant losses. Digestion of whole tissue with cyanogen bromide (CNBr) may overcome these problems since it results in an almost complete solubilization of collagen and requires minimal sample treatment (Cheung et al., 1990; Light, 1982). In addition, it results in collagen type-specific peptides (Bornstein, 1980). CNBr-derived collagen peptides have been analysed in lung chromatographically and/or electrophoretically (Kelley et al., 1989; Reiser and Last, 1980). However, these methods are cumbersome and of limited sensitivity (at best about 1  $\mu$ g). Minor

45°C. The CNBr stock solution contained 2 g/ml acetonitrile.

# Immunization and antibodies

To obtain antibodies which react specifically with type-specific CNBr-derived collagen peptides, Wistar rats (9, about 150g) and New Zealand white rabbits (3 or  $\Im$ , about 2 kg) were s.c. injected at different spots with either I mg CNBr-derived collagen peptides in 1 ml 0.9% (w/v) NaCl or with 1 mg collagen in 1 ml 0.5 M HAc, and mixed with 1 ml complete Freunds' adjuvant. After four weeks, animals were boostered with the same amount of antigen in incomplete Freunds' adjuvant. Animals were bled and the serum collected when the antibody titre did not further increase. Antibodies were screened for specificity by means of inhibition enzyme immunoassays (see below), using CNBr-derived collagen peptides.

# Sample preparation

The procedure of sample preparation is outlined in Fig. 1. Human lung parenchyma was powdered in a mortar precooled with liquid nitrogen, and lyophilized. One millilitre 70% (v/v) formic acid was added to 1 mg material and the solution was bubbled with nitrogen for 10 min. CNBr (2g/ml acetonitrile) was added to a final concentration of 8% (w/v) and incubation was for 2 hr at 45°C. After centrifugation at 5000 g for 10 min, a similar amount of CNBr in 70% formie acid was added to the pellet and incubation was repeated. After centrifugation at 5000 g for 10 min, the two supernatants were pooled and 10 Vol of deionized water was added. The material was lyophilized and subsequently dissolved in 10 mM Tris-HCl/0.9% (w/v) NaCl/0.05% Tween-20 (TBST) (pH 7.5) at a protein concentration of 2 mg/ml.

collagens can therefore not be analysed this way.

We here report a method to quantify different types of collagen in lung using immunoassays based on collagen type-specific antibodies reactive with CNBr-derived peptides.

## MATERIAL AND METHODS

Human lung specimens were obtained from lung lobes after lobectomy or pneumonectomy and immediately frozen in liquid nitrogen. The samples were taken from peripheral lung tissue that did not show any sign of the process for which the patient (age range 55-71 years) was operated.

collagen was estimated from the hydroxyproline from human placenta by limited pepsin digescontent by multiplying this value with a factor tion and selective salt precipitation according to 7, based on the observation that, on average, Miller and Rhodes (1982) and Abedin et al. 15% of the amino acids in collagen are hydroxy-(1981). CNBr-derived peptides were obtained by prolines (Last and Reiser, 1984). Amino acid treatment of collagen (1 mg/ml 70% (v/v))analysis was performed after hydrolysis under formic acid) with 2% (w/v) CNBr for 4 hr at vacuum in 6 M HCl at 100 °C for 24 hr using a

# Analytical methods

Hydroxyproline content was measured according to Huszar et al. (1980). Alkaline hy-Isolation of collagens drolysis was for 2 hr at 120°C. The amount of Type I, III, IV and V collagen were isolated

# Quantification of collagens in human lung

Varian LC 5000 HPLC amino acid analyser. Protein was determined according to Lowry et al. (1951). Sodium dodecyl sulfatepolyacrylamide gel electrophoresis was performed according to Laemmli (1970), using 10% gels and reducing conditions (i.e. 5% 2-mercaptoethanol).

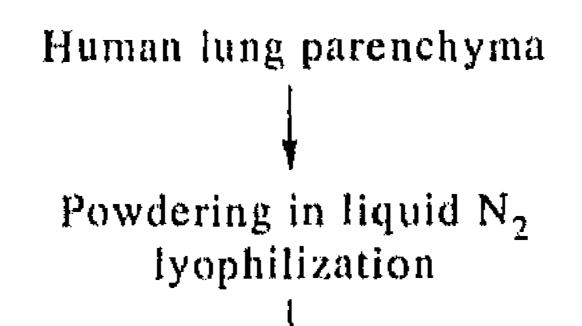
# Non-equilibrium inhibition immunoassay

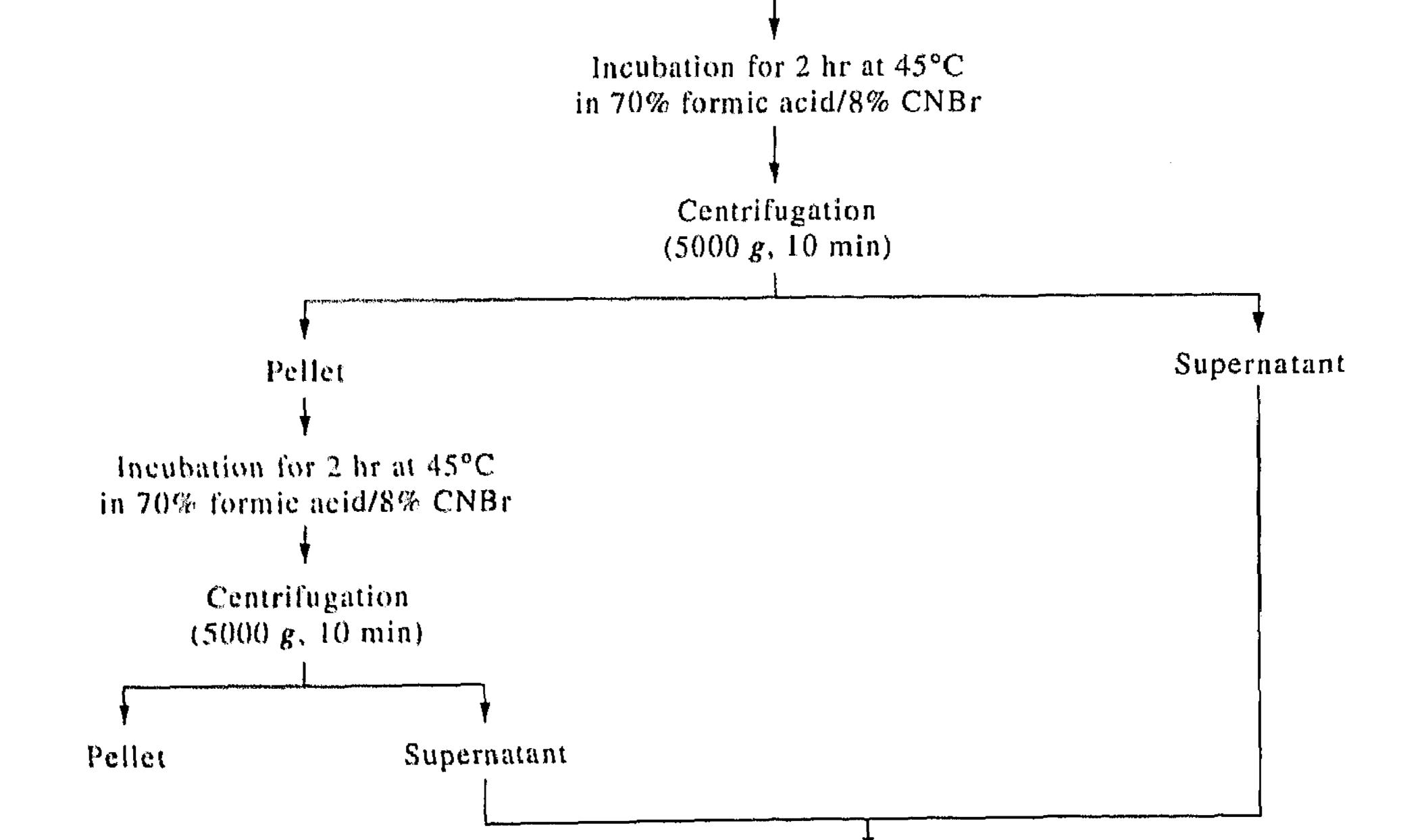
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Non-equilibrium inhibition assays were carried out according to Rennard *et al.* (1980). Wells of polystyrene microtitre plates were coated by incubation overnight at 4°C with

overnight at 4°C. One hundred microlitres of each well was transferred to a corresponding well of a polystyrene plate previously coated with CNBr-derived collagen peptides and incubated for 30 min at 22°C. Free antibodies, i.e. antibodies not bound by collagen in the sample, were allowed to complex with the coated CNBrderived collagen peptides. After washing 3 times with TBST, bound antibodies were detected by incubation for 1 hr at 20°C with 100  $\mu$ l rabbit anti-rat IgG (in case of rat sera) or goat antirabbit IgG (in case of rabbit sera) conjugated with peroxidase (1:1000 in TBST/BSA; Sigma, St Louis, Mo, U.S.A.). After washing, 100  $\mu$ l of substrate (0.04% (w/v) *o*-phenylenediamine and 0.016% (w/v) H<sub>2</sub>O<sub>2</sub> in 24 mM citric acid/51 mM  $Na_2HPO_4$  (pH 5.5)) was added. The reaction was allowed to proceed for 30 min at 22°C after which it was stopped by addition of 100  $\mu$ I 4 M H<sub>2</sub>SO<sub>4</sub>. The colour developed was spectrophotometrically measured at 492 nm. Each tissue digest was analysed at various dilutions in such a way that the data from at least two different dilutions were in the steep part of the standard curve.

100  $\mu$ l 20 mM carbonate buffer (pH 9.6) containing 500 ng CNBr-derived peptides of the appropriate type of collagen isolated from placenta. After incubation the wells were treated with 200  $\mu$ l 10 mM Tris-HCl, 0.9% (w/v) NaCl (pH 7.5) containing 1% (w/v) bovine serum albumin for 1.5 hr at 20°C and washed 3 times with TBST. In the wells of round-bottom polyvinyl microtitre plates 50  $\mu$ l of the serum, diluted 1:2000 in TBST containing 1% (w/v) bovine serum albumin (TBST/BSA; pH 7.5) was mixed with 100  $\mu$ l sample and incubated



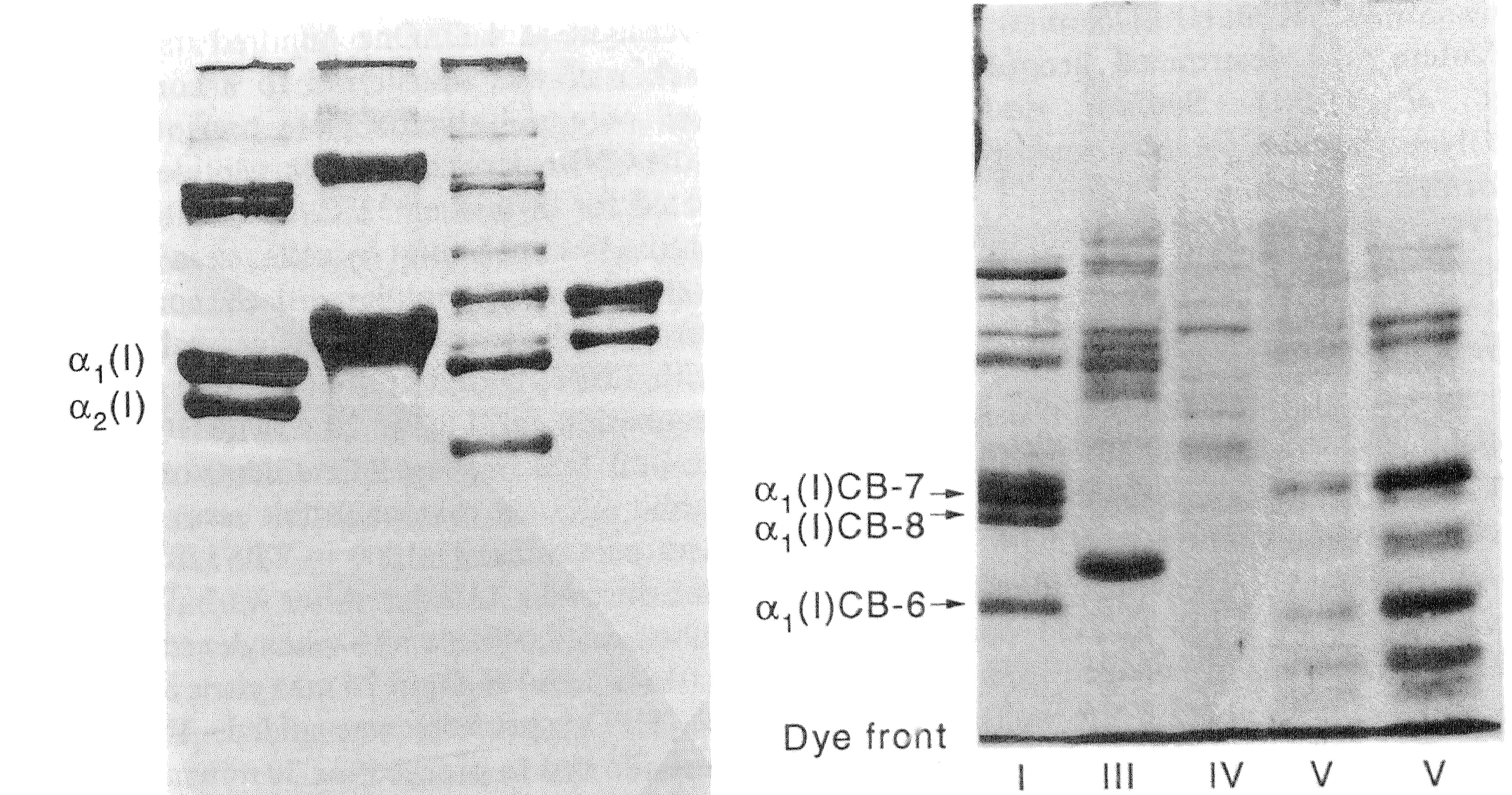


# Lyophilization and solubilization in TBST

Sample for immunoassays

Fig. 1. Preparation of human lung tissue for immunochemical analysis of collagen. TBST: 10 mM Tris-HCl/0.9% (w/v) NaCl/0.05% Tween-20.

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# Dye front

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of different types of collagens. Reducing conditions were used. Collagens were isolated from human placenta by limited pepsin digestion and selective salt precipitation. I: type I collagen; III: type III collagen; IV: type IV collagen; V: type V collagen. The  $\alpha_1$  and  $\alpha_2$  chains of collagen type I are indicated.

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of placenta collagens after cleavage with cyanogen bromide. Reducing conditions were used. Each type of collagen has its own unique set of cyanogen bromide peptides. I: type I collagen, III: type III collagen, IV: type IV collagen, V: type V collagen. The positions of three CNBr-derived peptides of type I collagen are indicated.

is  $20 \pm 4 \mu g/mg dry weight (mean \pm SD, n = 5)$ and the amount of hydroxyproline in the CNBr supernatants is 1845 ug/mg dry WCIGNI (n = 10). The remaining pellet has an amino acid composition which resembles that of clastin (i.e. high in alanine and valine, no methionine, >40% hydrophobic amino acid residues, about

# RESULTS

Isolation of collagens from human placenta

Isolation of collagen using limited pepsin digestion and differential salt precipitation results in collagen preparations which are essentially free of other proteins as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). The different types of collagen are not contaminated by one another. Even when the gel was grossly overloaded, no additional protein bands could be observed. In our hands, the method described by Abedin and coworkers (1981) was especially effective for the separation of type IV and V collagen. CNBr cleavage results in peptide fragments, characteristic for each type of collagen (Fig. 3). Results also indicate that contamination with other types of collagen is absent.

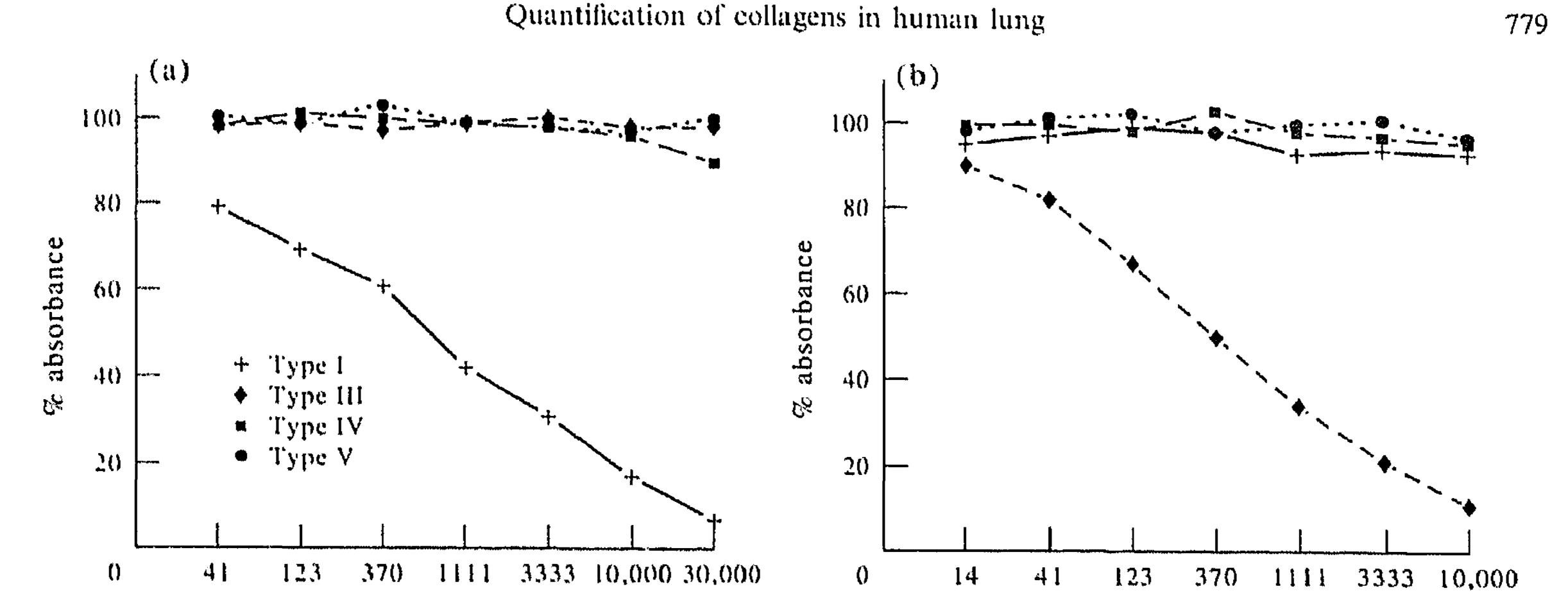
Table 1. Amino acid composition of the pellet obtained after CNBr digestion of human lung parenchyma

Amino acid	Number/1000 residués	
Aspartic acid asparts.		
Lydroxyproline		
Threonine		
Serinc		
Glutamic acid/glutamine		
Proline		
Cilvine		
Alanine		
Valine		
Cysteine		
Methionine		
Isoleucine		
Tyrosine		
Phenylalanine	and the second sec	
Histidine		
Lydroxylysine		
Arginine		

Extraction of collagen from human lung parenchyma

Direct digestion of lung tissue by CNBr results in an almost complete solubilization of collagen. The tissue content of hydroxyproline

Values are expressed as residues per 1000 residues and are not corrected for destruction during hydrolysis. nd: not determined.



CNBr-derived collagen peptides (ng)

CNBr-derived collagen peptides (ng)

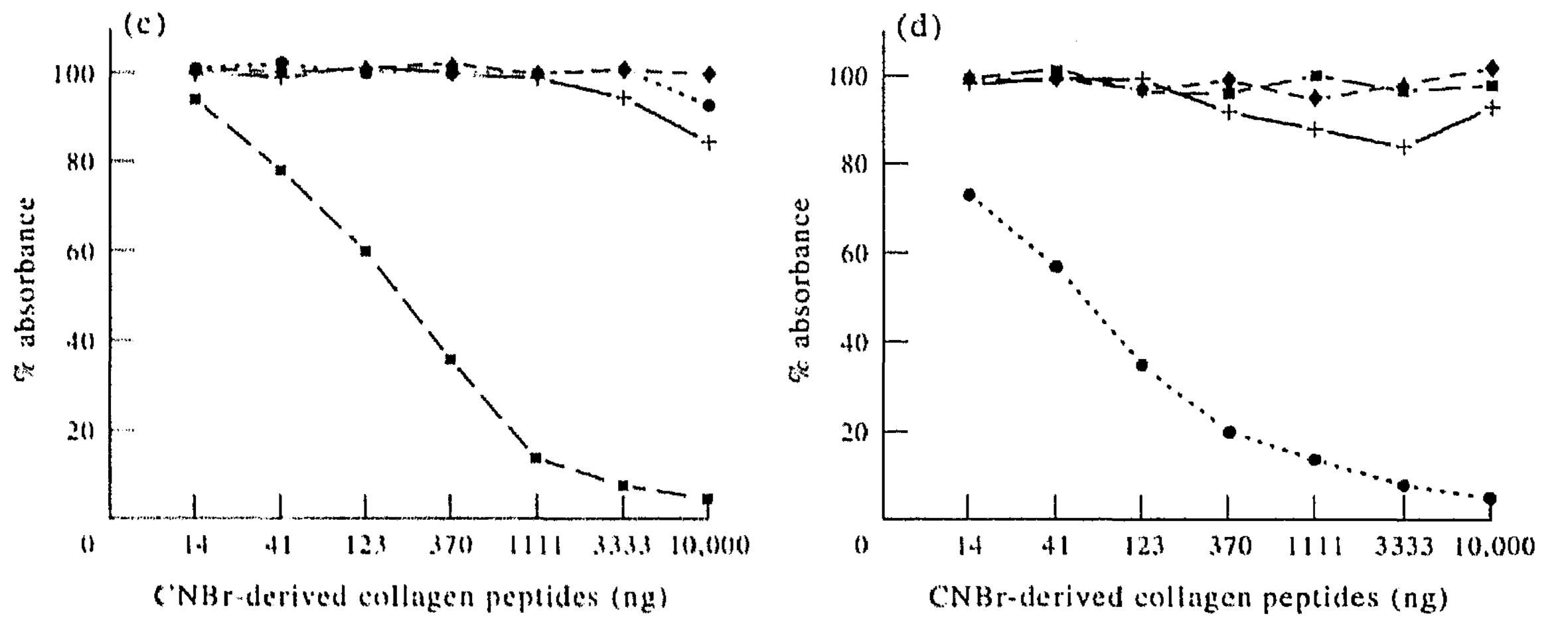


Fig. 4. Inhibition immunoassays for the quantification of type I, III, IV and V collagen. A volume of 100  $\mu$ l containing various amounts of CNBr peptides derived from different types of collagen were incubated

overnight at 4°C with 50  $\mu$ l of antibodies reactive with cyanogen bromide-derived peptides of type I (a), type II (b), type IV (c) and type V collagen (d). Next, 100  $\mu$ l of these mixtures were transferred to a corresponding well previously coated with 500 ng of CNBr-derived peptides of the appropriate type of collagen. After incubation for 30 min at 22°C, the antibodies bound to the well were detected by addition of peroxidase-conjugated secondary antibodies. After addition of substrate the colour developed was spectrophotometrically measured at 492 nm. The absorbance is expressed as a percentage of that without CNBr-derived peptides, which is taken as 100%. Type I, III, IV and V: CNBr-derived peptides from placenta collagen type I, III, IV and V, respectively.

one-third glycine and 10–13% proline) (Table 1). Elastin is not degraded by CNBr since it does not contain methionine residues. Hydroxylysine, characteristic for collagen, is absent from the pellet, as is methionine. Hydroxyproline residues constitute about 1.5% of the amino acid residues in the CNBr pellet; lung elastin contains about 1.4–1.7% hydroxyproline (Pierce and Hocott, 1960; Chrzanowski

Sepharose 4B to which CNBr-derived collagen peptides had been coupled did not alter specificity in any way. For collagen type I and III, sera of rabbits immunized with collagen were used. These sera react with CNBr-derived collagen peptides (Fig. 4a,b), like the anti-collagen antibodies previously described (Bellon, 1985; Srinivas et al., 1993). Antisera raised against CNBr-derived collagen fragments of type I and III collagen were not type-specific. For type IV and type V collagen, sera of rats immunized with CNBr-derived collagen peptides were used. These sera were type-specific (Fig. 4c,d). In the inhibition enzyme immunoassay for type I collagen, 50% inhibition was achieved with about  $1 \mu g$  (Fig. 4a); for type III collagen this value

et al., 1980).

Quantification of collagens

The antisera used for the quantification of CNBr-derived collagen peptides (and hence for collagen itself) are type-specific (Fig. 4). Immunoadsorption using CNBr-activated

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was about 350 ng, for type IV collagen about 200 ng and for type V collagen about 50 ng (Fig. 4b-d).

Using less than 1 mg of lyophilized parenchymal lung tissue all four collagens could be quantified (Table 2). Type I collagen represents 69% of the total collagen, type III 21%, type IV 8% and type V 1.5%. The amount of collagen estimated by hydroxyproline measurement equals the amount of collagen calculated by summing the amount of type I, III, IV and V collagen (Table 2). The ratio between the former and the latter is  $1.02 \pm 0.19$  (mean  $\pm$  SD, n = 10). This shows that collagen is quantitatively assayed by a combination of the immunoassays. while incomplete digestion and separation would influence electrophoretical and chromatographical analysis.

The results described here are in line with immunoassays reported for CNBr peptides from collagens in kidney and placenta (Bellon, 1985), chondrosarcoma cultures (Srinivas et al., 1993) and skin and aorta (Schröter-Kermani et al., 1990). In comparison with the immunoassays for types IV and V collagen, those for the types I and III collagens are less sensitive. This, however, is compensated by the relative abundance of these interstitial collagens. The steepness of the inhibition curve for type I collagen is relatively shallow and this will limit the accuracy of the assay. Others have found similar results for type I collagen immunoassays (e.g. Schröter-Kermani et al., 1990). In addition, the  $\alpha_3$  and  $\alpha_4$  chains of type IV collagen will not be analysed since type IV collagen from human placenta, which lacks these chains, was used as the antigen to prepare type IV specific antiserum. For human lung parenchyma, we found that type I collagen constitutes 69% of the total collagen, which is in agreement with data from the literature (66%, Seyer et al., 1976). The value for collagen type III of 21% is somewhat less than reported (33%, Seyer et al., 1976). In this study, however, other types than type I and III were not taken into consideration and whole lung instead of lung parenchyma was assayed. In parenchymal lung tissue from the rat, collagen type III represents 18% of the total collagen (Kelley et al., 1989), while this is 33% in whole lung (Last and Reiser, 1980). Type IV collagen (8% of the total collagen) has not been quantified before in lung. Lung parenchyma is rich in basement membranes (alveolar and endothelial basement membrane), to which type IV collagen is restricted. In other basement membrane-rich tissues type IV collagen also constitutes a considerable proportion

 $21\pm5$ 

 $8\pm 2$ 

 $1.5 \pm 0.3$ 

#### DISCUSSION

In this study we describe immunoassays to quantify different types of collagen, including minor collagens. There are three main advantages of analysing CNBr-derived collagen peptides immunochemically instead of chromatographically and/or electrophoretically, techniques that have been used for lung collagens previously (Laurent et al., 1981; Kirk et al., 1984). First, inhibition enzyme immunoassays are very sensitive: the detection limit of type IV collagen is about 40 ng, of type V collagen about 10 ng (Fig. 4). Second, various collagens (in this study type I, III, IV and V) can be analysed using the same sample. This is impossible using electrophoretic techniques since many peptides arise after CNBr digestion of whole tissue and they overlap each other (Bornstein, 1980). In particular, minor collagens give rise to many CNBr-derived peptides (e.g. each  $\alpha$ -chain of type IV collagen contains 32 methionine residues, which form the site of cleavage). Third, separation of the CNBr-derived peptides (often two or more peptides are still linked) is not necessary for immunochemical quantification,

Table 2. Content of type I, III, IV and V collagen and total collagen in human		
lung parenchyma		

Collagen	$\mu$ g/mg dry weight	% of total collagen
Type I	$84.6 \pm 16.1$	$69 \pm 4$

Type I $84.6 \pm 16.1$ Type III $26.6 \pm 10.3$ Type IV $9.6 \pm 2.0$ Type V $1.8 \pm 0.3$ Total collagen $122.6 \pm 23.2$ (type I, III, IV, V added) $124.0 \pm 36.0$ Total collagen $124.0 \pm 36.0$ 

Values are mean  $\pm$  SD (n = 10).

## Quantification of collagens in human lung

of the collagen, e.g. placenta 3-21% (Bellon, 1985; Garbisa *et al.*, 1980) and kidney 13% (Bellon and Borel, 1988). Type V collagen was estimated to constitute 1.5% of the collagen in human lung parenchyma. In total rat lung, type V collagen represents about 5% (Reiser and Last, 1983). In placental membranes a value of less than 3% was found (Miller *et al.*, 1991).

Since our method is very sensitive, collagens can be quantified in morphologically defined cryosections, as for instance in sections with only alveoli (where small vessels have been cut away) or in sections in which a pathological condition is prominent (e.g. fibrosis). A cryosection of  $20 \,\mu \text{m} \times 1 \,\text{cm} \times 1 \,\text{cm}$  contains about 15  $\mu$ g collagen (assuming 80% is air, 70% of the tissue is water, and 12% of the dry weight is collagen). This is sufficient collagen to quantify the different types using the immunoassays described here. In conclusion, we developed a technique for the quantification of different types of collagen in small tissue samples ( < 1 mg dry weight). The method is based on digestion of the tissue by CNBr and analysis of the resulting, type-specific peptides by inhibition enzyme immunoassays. The method combines simplicity (CNBr digestion is performed on whole tissue) with specificity and sensitivity (inhibition enzyme immunoassays). Minor collagens can be analysed

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