Proc. Nat. Acad. Sci. USA
Vol. 71, No. 7, pp. 2828–2832, July 1974

Lung Collagen Heterogeneity

(protein synthesis)

KATHRYN BRADLEY, SALLY McCONNELL-BREUL, AND RONALD G. CRYSTAL

Section on Pulmonary Biochemistry, National Heart and Lung Institute, Bethesda, Md. 20014

Communicated by Donald S. Fredrickson, April 18, 1974

ABSTRACT **The structural heterogeneity of rabbit lung collagen was examined by extracting labeled collagen from short-term cultures of lung minces with 1 M NaCl–50 mM Tris·HCl (pH 7.4), 0.5 M acetic acid, or 0.4 ionic strength phosphate buffer. The extracted collagens were purified by carboxymethyl-cellulose chromatography, and their cyanogen bromide peptides were mapped by ion exchange chromatography and acrylamide gels. Rabbit skin α1(I) and α2 chains and rabbit sternal cartilage α1(II) chains were used as markers.**

The peripheral lung, containing alveoli, small blood vessels, and small airways, synthesized α1(I) and α2 chains. The trachea and the bronchial tree (first through seventh order branches) both synthesized α1(II) chains. Lung α1(I), α2, and α1(II) chains all have a molecular weight of about 100,000 and are all sensitive to Clostridial collagenase.

The extraction and purification methods used isolate only 50% of the collagen synthesized by these structures *in vitro*. Once all collagen types in lung can be described and quantitated, it should be possible to utilize lung collagen types as biochemical markers to study normal lung development and to define the lung fibrotic diseases.

The connective tissue of the lung is fundamental to the growth, development, and ultimate structure and function of this organ. Implicit in an understanding of the mechanisms involved in the development of lung structure is an understanding of the connective tissue matrix comprising it. Collagen is the most abundant protein of this matrix, and it comprises 10% of the dry weight of adult lung (1). Morphologic studies have demonstrated collagen in association with the tracheobronchial tree, blood vessels, and alveolar interstitium (2, 3). Some of this collagen can be extracted from rabbit or human lung by conventional techniques, and its amino-acid composition is similar to that of collagen from other sites in the body (1).

Several structurally distinct collagen chains have been described in other organs (4). The most completely described is acid-soluble collagen found in skin, tendon, and bone. Ths collagen is composed of two chains, α1(I) and α2, making up a tropocollagen molecule of composition $[\alpha 1(I)]_2\alpha 2$ (5–10). In cartilage, α1(II) (tropocollagen $[\alpha 1(II)]_3$) predominates (11–17). In human skin, uterine leiomyoma, and aorta, α1-(III) (tropocollagen $[\alpha 1(III)]_3$) has been found (18, 19). In addition, another type, α1(IV) (presumed tropocollagen $[\alpha 1(IV)]_3$), has been described in basement membrane from the renal glomerulus and lens capsule (20, 21).

Abbreviation: CM-cellulose, carboxymethyl-cellulose.

In tissue culture, specific cell types have been associated with the synthesis of specific collagen types. For example, fibroblasts derived from skin synthesize α1(I) and α2 (22, 23) and epithelial cells derived from lens capsule synthesize α1(IV) (24). The lung theoretically may have all types of collagen present, since it is a complex organ comprised of more than forty cell types (3). This study demonstrates the synthesis by lung of three structurally distinct collagen α chains. Two of these chains, α1(I) and α2, appear identical to skin α1(I) and α2; and the third chain, α1(II), appears identical to sternal cartilage α1(II).

MATERIALS AND METHODS

New Zealand white rabbits, all from an inbred strain, were used as the source of lung, skin, and cartilage. Skin α1(I) and α2 chains were extracted and purified as described (1, 25). Cartilage α1(II) chains were extracted from ribs and sternae of 9-week-old lathyritic rabbits (17).

Lung Collagen Synthesis In Vitro. Lungs were removed from exsanguinated rabbits and washed in phosphate-buffered saline (pH 7.4) at 4°. Three types of incubations were done, each under the same conditions and each using ten 3-week-old rabbits but with different lung structures. Peripheral lung preparations consisted of the lung remaining after removal of the major airways and blood vessels. Tracheal preparations consisted of the trachea from just below the larynx to the carina. Bronchial preparations consisted of the bronchial tree (first through seventh order bronchi) dissected free from surrounding tissues with the aid of a microscope. In each case, the material to be incubated was minced with scissors and the mince was washed with incubation medium (equal parts Dulbecco's modified Eagle's medium and phosphate-buffered saline; 0.5 mM ascorbic acid; 0.7 mM β-aminopropionitrile, BAPN) (1). The mince was then incubated in fresh medium for 45 min at 37° under 95% O_2–5% CO_2. This medium was aspirated, replaced with fresh medium containing [5-^{3}H]proline (5–13 μM, 25 Ci/mmole), [^{3}H]glycine (5–13 μM, 33 Ci/mmole), or both, and the incubation with isotope continued for 4 more hours. After incubation, the collagen synthesized *in vitro* was extracted (1) in the buffers described below.

Extraction and Purification of α1(I) and α2 Synthesized In Vitro from Peripheral Lung. The labeled collagen was extracted from peripheral lung with 0.5 M acetic acid for 24 h at 4°. The insoluble material was discarded, and the supernatant dialyzed against 0.5 M acetic acid. The collagen was precipitated from the supernatant with 5% (w/v) NaCl, and

labeled α1(I) and α2 chains were isolated on a carboxymethyl (CM)-cellulose column as described for the skin α1(I) and α2 chains (1, 25). Alternatively, the labeled α1(I) and α2 chains were extracted from peripheral lung with 0.4 ionic strength phosphate buffer (26) and subsequently chromatographed on CM-cellulose as described above.

Extraction and Purification of α1(II) Synthesized In Vitro from Trachea and the Bronchial Tree. From the incubations of trachea or bronchi, the labeled collagen was extracted by stirring for 24 hr at 4° in 1 M NaCl–50 mM Tris·HCl (pH 7.4). The insoluble material was discarded and the supernatant was dialyzed against 20 mM sodium acetate, pH 4.8–1 M urea before chromatography on CM-cellulose as described for cartilage α1(II) chains (17). Alternatively, after extraction, the tracheal preparations were dialyzed against 0.2 M NaCl–50 mM Tris·HCl, pH 7.4 for 12 hr at 4° and applied to a diethylaminoethyl (DEAE)-cellulose column equilibrated with the same buffer at 4°. The labeled α1(II) chains were eluted with the equilibrating buffer (13) and further purified on CM-cellulose as described above. Tracheal and bronchial α1(II) chains could also be extracted with 0.4 ionic strength phosphate buffer as described for cartilage α1(II) (26).

Purified labeled α1(I), α2, and 1(II) chains were subjected to electrophoresis on 5% sodium dodecyl sulfate–acrylamide gels (1, 27). After electrophoresis, the gels were either stained and scanned (1) or fractionated and counted in Aquasol (New England Nuclear Corp.) with an efficiency of 30%. Sensitivity of labeled lung collagen chains to purified Clostridial collagenase was tested as described (1, 28).

Cyanogen Bromide Cleavage and Peptide Mapping. Cyanogen bromide (CNBr) cleavage was performed on purified skin, cartilage, peripheral lung, tracheal, or bronchial α chains dissolved in 70% (w/v) formic acid (18). The resulting peptides were freed of reagent and formic acid by passing the reaction mixture over a column of Biogel P2 (100–200 mesh) equilibrated with 0.1 M acetic acid. The fractions containing the peptides were lyophilized to dryness, and stored at −20°.

Separation of the peptides was done on a 1.5 × 5-cm column of CM-cellulose equilibrated with sodium citrate (75 mM, pH 3.8) at 43° and eluted at 50 ml/hr with a linear NaCl gradient (0.02–0.2 M, 500 ml total) (7). The major peptides were pooled, chromatographed on a Biogel P2 column in 0.1 M acetic acid (7), and lyophilized to dryness.

These individual peptides were then subjected to electrophoresis on 7.5% dodecyl sulfate–acrylamide gels (5 hr, 8 mA per gel). After electrophoresis, the gels were either stained and scanned or fractionated and counted in Aquasol as described above. Aliquots of the original peptide mixture were subjected to electrophoresis in parallel gels.

RESULTS

The peripheral lung synthesized at least two collagen chains *in vitro*. These can be extracted with 0.5 M acetic acid and separated on CM-cellulose under the identical conditions used to separate skin α1(I) and α2 (5, 6, 25). Peripheral lung α1 and skin α1(I) cochromatographed as did peripheral lung α2 and skin α2 (Fig. 1*A*). When peripheral lung collagen was extracted with 0.4 ionic strength phosphate buffer, pH 7.6, the result on a CM-cellulose column was similar.

The trachea synthesized at least one collagen chain *in vitro*. When this material was extracted with 1 M NaCl–20 mM Tris·HCl, pH 7.4, and chromatographed on DEAE-cellulose at 4°, a large peak of radioactive labeled protein eluted with 0.2 M NaCl. When this material was subsequently chromatographed on CM-cellulose, only one peak was seen, chromatographing with sternal cartilage α1(II) chains (Fig. 1*B*). If the DEAE-cellulose purification step was omitted, there was still only one peak on subsequent CM-cellulose chromatography. If the trachea was extracted with 0.5 M acetic acid or with 0.4 ionic strength phosphate buffer, there was still only one peak on subsequent CM-cellulose chromatography. The same extraction procedures demonstrated one collagen chain synthesized by the bronchial tree (Fig. 1*C*).

Extraction of the *in vitro* product of minces of whole lung (including peripheral lung and the bronchial tree) with 0.5 M acetic acid or 0.4 ionic strength phosphate buffer yielded a variable ratio of α1 to α2 chains on CM-cellulose (α1/α2 >

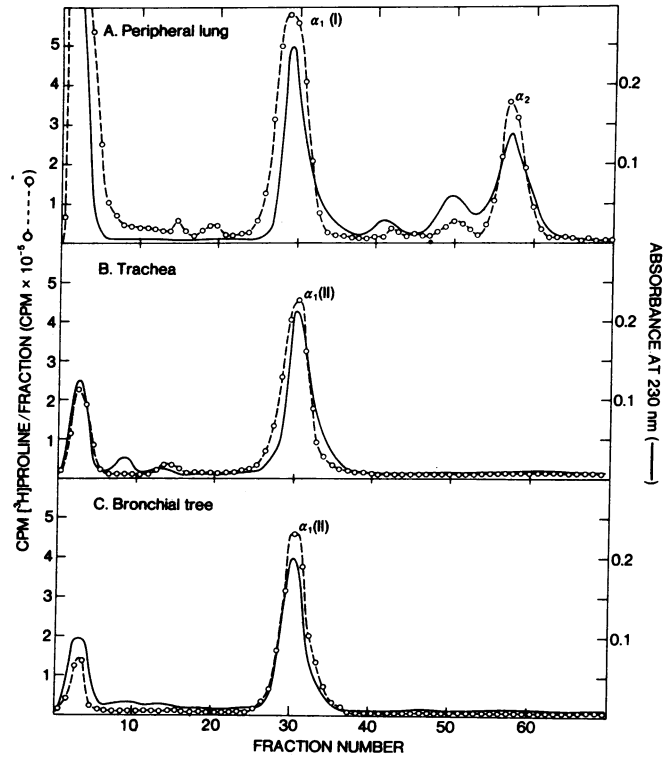

FIG. 1. CM-cellulose chromatography of rabbit lung collagen chains synthesized *in vitro*. (*A*) After incubation with [³H]proline, peripheral lung collagen was extracted with 0.5 M acetic acid and precipitated with 5% NaCl before chromatography. Lathyritic rabbit skin collagen (10 mg) extracted with 1 M NaCl–20 mM Tris·HCl, pH 7.4, was used as carrier (see *Materials and Methods*). (*B*) After incubation with [³H]proline, tracheal collagen was extracted with 1 M NaCl–20 mM Tris·HCl, pH 7.4. This material was first chromatographed on DEAE-cellulose at 4°. The material in the radioactive peak eluting with 0.2 M NaCl was dialyzed against 20 mM sodium acetate, pH 4.8, denatured at 45° for 20 min, and then chromatographed as shown here. Sternal cartilage α1(II) (10 mg) was used as carrier. (*C*) After incubation with [³H]proline, bronchial collagen was extracted with 0.4 ionic strength phosphate buffer and precipitated with 2.2 M NaCl. The precipitate was solubilized in 20 mM sodium acetate, pH 4.8, and chromatographed with 10 mg of sternal cartilage α1(II) chains as carrier. Columns *A–C* were run under identical condition (see *text*). Ten-milliliter fractions were collected and counted in Aquasol with an efficiency of 30%. cpm/fraction (O) and absorbance at 230 nm (*solid line*) are shown.

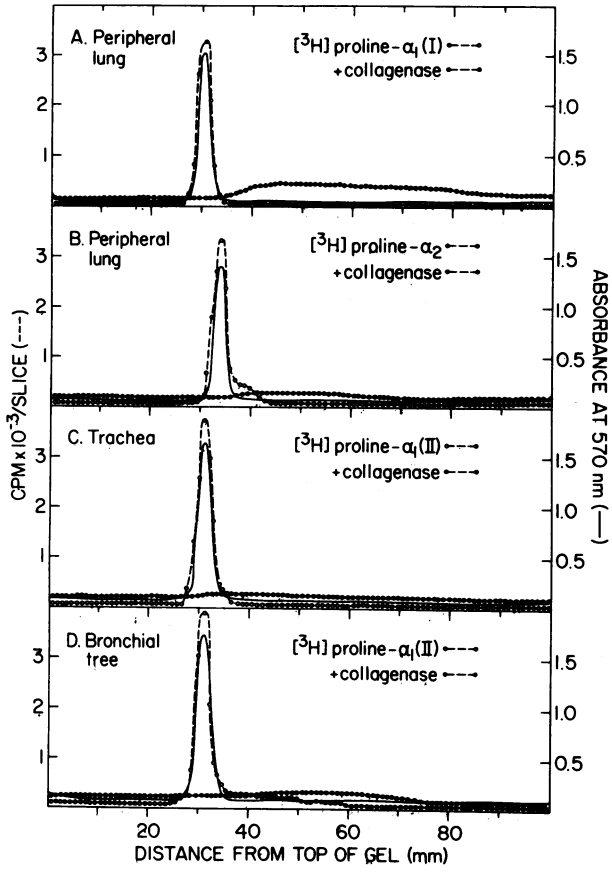

FIG. 2. Sodium dodecyl sulfate–acrylamide gel electrophoresis of rabbit lung collagen chains synthesized *in vitro*. The gels were subjected to electrophoresis for 6 hr at 8 mA per gel. (*A*) α1(I) chains synthesized by peripheral lung (O) electrophoresed with rabbit skin α1(I) chains (*solid line*). Exposure to Clostridial collagenase electrophoresis hydrolyzed the lung α1(I) chains (●). (*B*) α2 chains synthesized by peripheral lung (O); preincubation with collagenase (●); with rabbit skin α2 chains as a marker (*solid line*). (*C*) α1(II) chains synthesized by trachea. These chains electrophoresed with rabbit sternal cartilage α1(II) chains (*solid line*) and were hydrolyzed by collagenase (●). (*D*) Identical to (*C*) but with α1(II) chains synthesized by the bronchial tree.

2). Presumably, the increased amount of α1 chains was secondary to a mixture of α1(I) and α1(II) (or other) chains eluting at the α1 position. Attempts to use the technique of Trelstad *et al.* (26) to identify α1(I) and α1(II) from this peak yielded radioactive peaks (containing hydroxyproline) in the positions described by these authors for α1(I) and α1(II) chains. However, the recovery of labeled lung α1(I) and α1(II) chains from this column was poor and only some of the recovered material was still intact α1(I) and α1(II) chains (data not shown).

The α1(I) and α2 chains synthesized by peripheral lung both have molecular weights of about 100,000 (Figs. 2*A* and *B*). These chains electrophoresed with skin α1(I) and α2 chains, respectively, and they were completely destroyed by Clostridial collagenase. The α1(II) chains synthesized by trachea or bronchi also have a molecular weight of 100,000 and were sensitive to Clostridial collagenase (Fig. 2*C* and *D*).

Further identification of peripheral lung α1 as α1(I) was done by CNBr peptide mapping on CM-cellulose in sodium citrate and on 7.5% dodecyl sulfate–acrylamide gels. The peptide pattern on CM-cellulose of lung α1(I) was similar to that of rabbit skin α1(I) (Fig. 3*A*). Although the CNBr peptides of rabbit skin α1 and α2 have been only partially described (29), the absorbance pattern on CM-cellulose was so similar to the pattern of rat skin α1(I) (5), human skin α1(I) (6), and chick bone α1(I) CNBr peptides (9), that the peaks were arbitrarily numbered in an analogous fashion. Amino acid analysis of lung α1 chains extracted with 0.5 M acetic acid [most likely α1(I) chains as the ratio of α1 to α2 was 2:1 (1)] has demonstrated 8 methionine residues/1000 residues. This would yield 9 CNBr peptides, similar to those described for other α1(I) chains. Rabbit skin α1 chains also have 8 methionine residues (29).

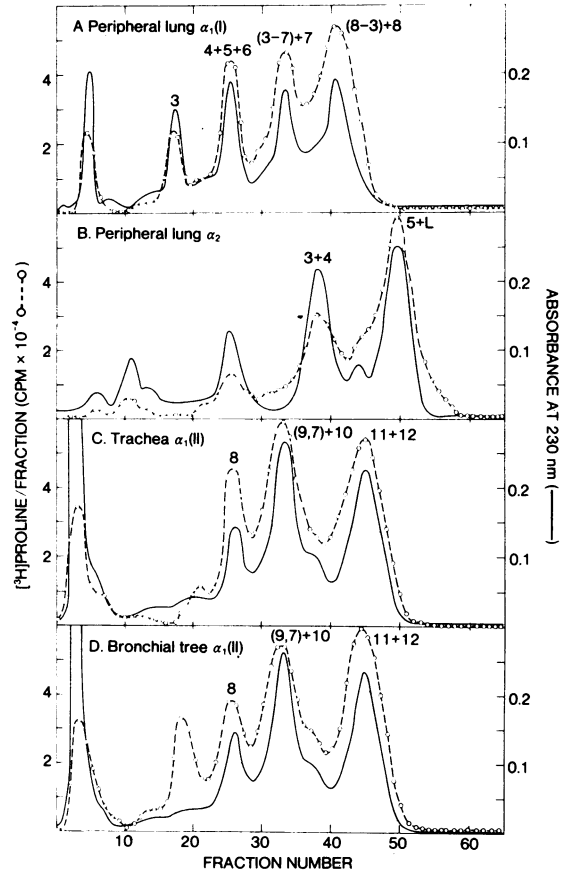

FIG. 3. CM-cellulose chromatography of cyanogen bromide peptides of rabbit lung collagen chains synthesized *in vitro*. (*A*) [3H]Proline-peripheral lung α1(I) peptides isolated from the α1 chains of Fig. 1*A*. The carrier was CNBr peptides of rabbit skin α1(I). (*B*) [3H]Proline-peripheral lung α2 peptides isolated from the material in the α2 peak in Fig. 1*A*. The carrier was CNBr peptides of rabbit skin α2. The peak labeled *5 + L* contains two components, one of molecular weight 57,000, the other of molecular weight 25,000 (see Fig. 4*B*). (*C*) [3H]Proline-tracheal α1(II) peptides isolated from the material in the α1(II) peak in Fig. 1*B*. The carrier was CNBr peptides of rabbit sternal cartilage α1(II). (*D*) [3H]Proline-bronchial α1(II) peptides isolated from the α1 chains of Fig. 1*C*. The carrier was CNBr peptides of rabbit sternal cartilage α1(II). Columns *A–D* were run in an identical fashion. Five-milliliter fractions were collected, and aliquots were counted in Aquasol. cpm/fraction (O) and absorbance at 230 nm (*solid line*) are shown. Identification of the CNBr peptides was made by analogy to known α1(I), α2, and α1(II) CNBr peptides (see *Results*).

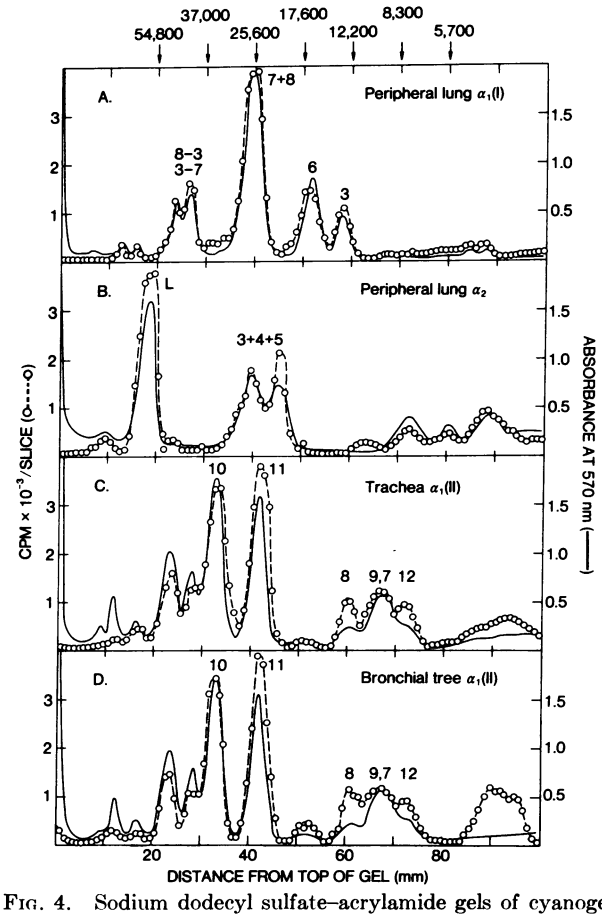

FIG. 4. Sodium dodecyl sulfate–acrylamide gels of cyanogen bromide peptides of rabbit lung collagen chains synthesized *in vitro*. The peptides placed on gels *A–D* were identical to those chromatographed on CM-cellulose (Fig. 3*A–D*). All gels were subjected to electrophoresis under identical conditions (6 hr, 8 mA per gel). (*A*) [^{3}H]Proline-peripheral lung α1(I) peptides with rabbit skin α1(I) peptides as carrier; (*B*) [^{3}H]proline-peripheral lung α2 peptides with rabbit skin α2 peptides as carrier. The peptide labeled *L* is probably peptide 3–5 (see *text*); (*C*) [^{3}H]proline-tracheal α1(II) peptides with rabbit sternal cartilage α1(II) peptides as carrier; and (*D*) [^{3}H]proline-bronchial α1(II) peptides with rabbit sternal cartilage α1(II) peptides as carrier. cpm/mm of gel (O) and absorbance of stained gels at 570 nm (*solid line*) are shown. Identification of CNBr peptides was made by isolating the major peptides of Fig. 3 and subjecting them to electrophoresis on individual gels in parallel (see *Results*). The molecular weight estimates (*top* of figure) were made with known skin α1(I) CNBr peptides.

The CNBr peptides of peripheral lung α1 and rabbit skin α1(I) also coelectrophoresed on dodecyl sulfate–acrylamide gels (Fig. 4*A*). The identification of the major peptides was made by isolating the major peaks from the CM-cellulose CNBr peptides and subjecting them to electrophoresis separately on parallel dodecyl sulfate gels. By analogy to the known sequence of bone α1(I) CNBr peptides (30, 31), a putative identification of the larger, probably uncleaved, peptides has been made (CNBr 8-3 and 3-7, Fig 4*A*). The molecular weight estimates (Fig. 4, top of figure) of the major peripheral lung α1(I) CNBr peptides agree closely with the known molecular weights of collagen CNBr peptides subjected to electrophoresis in parallel gels. By the criteria used, it appears that the α1 chains synthesized by peripheral lung are very similar, if not identical, to skin α1(I) chains.

Likewise, the CNBr peptides of the α2 chains synthesized by peripheral lung were very similar to rabbit skin α2 peptides (Fig. 3*B* and 4*B*). In all α2 chains that have been closely examined [including newborn rabbit lung α2 chains (1, 4)], there are 5 methionine residues/1000 residues. For that reason, the CNBr peptides mapped on CM-cellulose have been arbitrarily numbered as shown. When these peptides were individually recovered from this column and subjected to electrophoresis on dodecyl sulfate–acrylamide gels, the peptide eluting last on CM-cellulose gave two major peaks, one of a molecular weight of about 57,000 (labeled *L* in the figure), the other of about 25,000. By analogy to sequence of known skin α2 CNBr peptides, the larger peptide is probably the result from an uncleaved methionine between CNBr 3 and 5. CNBr cleavage of bone α2 also results in higher molecular weight peptides, presumably from incomplete cleavage (see Fig. 3 in ref. 30). It is possible, however, that the rabbit lung and α2 peptides may have a methionine missing, as has been described for pig skin α2 chains (32).

The α1 chains synthesized by trachea and bronchi were identified as α1(II) chains when the CNBr peptides of these chains were chromatographed on CM-cellulose (Fig. 3*C* and *D*) and dodecyl sulfate–acrylamide gels (Fig. 4*C* and *D*). By these criteria, lung α1(II) chains are very similar to rabbit sternal cartilage α1(II) chains. The CNBr designations on CM-cellulose are by analogy to bovine and human cartilage α1(II) CNBr peptides (16). The molecular weight estimates on dodecyl sulfate–acrylamide gels were as expected for these peptides. While the identities of CNBr 8, 10, 11, and 12 are clear by these criteria, that of CNBr (9, 7) is only by analogy to elution position and molecular weight estimate of bovine and human α1(II) CNBr (9, 7) (16). We do not know the identity of the peptide eluting on CM-cellulose before CNBr 8, although its molecular weight is about 9,000–11,000.

DISCUSSION

The finding of α1(II) chains in the lung tracheobronchial tree is expected since there is morphological evidence of cartilage from the trachea to ninth order bronchi (3). The finding of α1(I) and α2 chains in the lung, while not unexpected (1), is interesting since, besides skin, they have been fully characterized only in bone and tendon. Chung and Miller have noted α1(I) and α2 chains in human aorta (19), and we have noted them synthesized by rabbit pulmonary arteries and veins *in vitro* (data not shown). Whether the α1(I) and α2 chains synthesized by peripheral lung were synthesized by blood vessels present in peripheral lung and/or were synthesized by cells in the alveolar interstitium is not known.

Early in our observations on the synthesis of lung collagen *in vitro*, two facts stood out: (*1*) the conventional techniques used to extract collagen extract variable amounts of the lung collagen synthesized *in vitro* and (*2*) only a portion of extracted lung collagen will appear on a CM-cellulose column under conventional conditions. About 40–50% of the labeled hydroxyproline present in an incubation of lung mince will appear on a CM-cellulose column by these methods. The composition of the "insoluble" collagen is not known. Preliminary attempts to identify these components by CNBr peptide maps on CM-cellulose and dodecyl sulfate–acrylamide gels have been thwarted by the complexity of the resulting

patterns. They may be α1(I), α2, and/or α1(II) chains that are made insoluble by association with other lung components such as proteoglycans (13, 33). Alternatively, the "insoluble" collagen may represent additional types of collagen chains that are not solubilized by the methods used. For example, although the trachea appears to synthesize only α1(II) chains it could synthesize α1(I), α2, α1(III), and/or α1(IV) chains that were not solubilized under the conditions used to demonstrate α1(II) synthesis.

The heterogeneity of lung collagen provides biochemical markers to follow collagen gene expression during normal lung development. For example, morphologic evidence suggests that tracheobronchial tree development is completed early in fetal life, followed by blood vessel and finally alveolar development (34). With a major biochemical component of these tissues now defined, it should be possible to study the control of formation of these structures.

The fibrotic lung disorders represent 20% of the noninfectious diseases of the lung (35). It is a heterogeneous group of disorders with many etiologies including inorganic dusts, organic antigens, physical injury, infection, and in association with systemic disease. Although these disorders stem from a broad group of primary insults, at least part of the lung's response in all these disorders includes the synthesis of collagen (35). The fact that lung collagen itself is heterogeneous suggests the possibility that the fibrotic lung disorders may be grouped with respect to the relative amount of types of collagen being synthesized. For example, the fibrosis resulting from airway injury may include an overproduction of α1(II), while the fibrosis resulting from systemic disease may include an overproduction of α1(I) and α2. A similar approach has been useful in examining the types of collagen synthesized by osteoarthritic cartilage (36), embryonic notochord (37), and osteogenic cartilage (38).

Because of the complexity of the lung, this will be possible only when methods can be developed to quantitate the amount of each chain synthesized. Preliminary experiments have suggested that the combination of a denaturing agent and a reducing agent, such as that used by Grant *et al.* to extract lens capsule collagen (39), will extract all the labeled hydroxyproline from a lung mince incubation. It is most likely that additional collagen types will be demonstrated in the lung once methods can be developed to extract them intact.

We thank Drs. J. Collins, A. Hance, R. Shulman, and G. Martin for their help and advice and Ms. Diana Winters for editing the manuscript.

1. Bradley, K. H., McConnell, S. D. & Crystal, R. G. (1974) *J. Biol. Chem.* **249**, 2674–2683.
2. Low, F. N. (1971) *Arch. Int. Med.* **127**, 847–852.
3. Nagaishi, C. (1972) *Functional Anatomy and Histology of the Lung* (University Park Press, Baltimore, Md.).
4. Gallop, P. M., Blumenfeld, O. O. & Seifter, S. (1972) *Ann. Rev. Biochem.* **41**, 617–672.
5. Butler, W. T., Piez, K. A. & Bornstein, P. (1967) *Biochemistry* **6**, 3771–3780.
6. Click, E. M. & Bornstein, P. (1970) *Biochemistry* **9**, 4699–4706.
7. Epstein, E. H., Scott, R. D., Miller, E. J. & Piez, K. A. (1971) *J. Biol. Chem.* **246**, 1718–1724.
8. Bornstein, P. (1967) *Biochemistry* **6**, 3082–3093.
9. Miller, E. J., Lane, J. M. & Piez, K. A. (1969) *Biochemistry* **8**, 30–39.
10. Nold, J. G., Kang, A. H. & Gross, J. (1970) *Science* **170**, 1096–1098.
11. Miller, E. J. & Matukas, V. J. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 1264–1268.
12. Miller, E. J., Woodall, D. L. & Vail, M. S. (1973) *J. Biol. Chem.* **248**, 1666–1671.
13. Miller, E. J. (1971) *Biochemistry* **10**, 1652–1659.
14. Strawich, E. & Nimni, M. E. (1971) *Biochemistry* **10**, 3905–3911.
15. Deshmukh, K. & Nimni, M. E. (1973) *Biochemistry* **133**, 615–622.
16. Miller, E. J. & Lunde, L. G. (1973) *Biochemistry* **12**, 3153–3159.
17. Trelstad, R. L., Kang, A. H., Igarashi, S. & Gross, J. (1970) *Biochemistry* **9**, 4993–4998.
18. Miller, E. J., Epstein, E. H., Jr. & Piez, K. A. (1971) *Biochem. Biophys. Res. Commun.* **42**, 1024.
19. Chung, E. & Miller, E. J. (1974) *Science* **183**, 1200–1201.
20. Kefalides, N. A. (1971) *Biochem. Biophys. Res. Commun.* **45**, 226–234.
21. Kefalides, N. A. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1151–1158.
22. Layman, D. L., McGoodwin, E. B. & Martin, G. R. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 454–458.
23. Smith, B. D., Byers, P. H. & Martin, G. R. (1972) *Proc. Nat. Acad. Sci USA* **69**, 3260–3262
24. Grant, M. E., Schofield, J. D., Kefalides, N. A. & Prockop, D. J. (1973) *J. Biol. Chem.* **248**, 7432–7437.
25. Piez, K. A., Eigner, E. A. & Lewis, M. S. (1963) *Biochemistry* **2**, 58–66
26. Trelstad, R. L., Kang, A. H., Toole, B. P. & Gross, J. (1972) *J. Biol. Chem.*, **247**, 6469–6473.
27. Weber, K. & Osborn, M. J. (1969) *J. Biol. Chem.* **244**, 4406–4412.
28. Peterkofsky, B. & Diegelmann, R. (1971) *Biochemistry* **10**, 888–994.
29. Bornstein, P. & Nesse, R. (1970) *Arch. Biochem. Biophys.* **138**, 443–450.
30. Vuust, J. & Piez, K. A. (1970) *J. Biol. Chem.* **245**, 6201–6207.
31. Vust, J. & Piez, K. A. (1972) *J. Biol. Chem.* **247**, 856–862.
32. Heinrich, W, Lange, P. M., Stirtz, T., Iancu, C. & Iteidemann, E. (1971) *FEBS Lett.* **16**, 63–67.
33. Öbrink, B. (1973) *Eur. J. Biochem.* **33**, 387-400.
34. Emery, J. (ed) (1969) "The Anatomy of the Developing Lung," *William Heinemann Medical Books Ltd.* (Lavenhaum Press Ltd., Suffolk, England).
35. *Respiratory Diseases Task Force Report* (1972) DHEW Publication No. (NIH) 73–432.
36. Nimni, M. & Deshmukh, K. (1973) *Science* **181**, 751–752.
37. Linsenmayer, T. F., Trelstad, R. L. & Gross, J. (1973) *Biochem. Biophys. Res. Commun.* **53**, 39–45.
38. Linsenmayer, T. F., Trelstad, R. G., Toole, B. P. & Gross, J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 870–876.
39. Grant, M. E., Kefalides, N. A. & Prockop, D. J. (1972) *J. Biol. Chem.* **247**, 3545–3551.