Identification of the Collagenous Proteins Synthesized by Cultured Cells from Human Skin†

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ABSTRACT: Cells cultured from human skin synthesize precursor forms of types I and III collagens. After denaturation and reduction, the polypeptide chains obtained from these molecules had molecular weights estimated to be 140,000 and 120,000. The larger chains, the pro α chains, are believed to be derived from the original precursor molecules. The smaller chains arise from altered forms, p-collagens, that may be normal intermediates in the conversion of both procollagens to collagens I and III.

In vivo, the biosynthetic precursors of collagen are short lived because they are efficiently converted to collagen (Martin et al., 1975). In contrast, this conversion is inefficient in cell cultures so that precursor forms of collagen accumulate in the medium (Layman et al., 1971). Initially, identification of medium proteins as precursors of collagens was based on their greater solubility in physiological solutions, on the demonstration of components of greater size than α chains in the denatured protein, and on the conversion of these proteins to collagen by limited proteolytic digestion (Layman et al., 1971). Subsequently, it was demonstrated that the chains in the collagenous medium proteins were largely disulfide linked (Burgeson et al., 1972; Smith et al., 1972) and that the reduced chains could be resolved by electrophoresis into four components with molecular weights ranging from 110,000 to 140,000 (Goldberg et al., 1972). Since type 1 collagen contains both α1 and α2 type chains, Goldberg et al. (1972) concluded that two of the medium chains were precursors of α1 and two precursors of α2. Similarly sized components found during studies of collagen biosynthesis (see Martin et al., 1975) are believed to arise from the intact precursor, procollagen, containing proα1(I) and proα2 chains of about 140,000 molecular weight and p-collagen, a smaller form produced from procollagen containing p-α1(I) and p-α2 chains of about 115,000 molecular weight.

Four genetically distinct collagens have so far been identified in various tissues. Type I collagen contains two α1(I) chains and one α2 chain while types II, III, and IV contain only a single chain species. Smith et al. (1972) separated the medium proteins synthesized by fibroblasts into three fractions by DEAE-cellulose chromatography and identified the major fraction as the precursor of type I collagen. Church et al. (1973) identified one fraction isolated from DEAE-cellulose as the precursor of a different collagen, an $(\alpha 1)_3$ molecule. Here we demonstrate that human fibroblasts synthesize procollagen types I and III.

Materials and Methods

Cells grown from biopsies of human skin were obtained from the American Type Culture Collection, Rockville, Md., and maintained in Dulbecco-Vogt medium supplemented with 10% fetal calf serum, penicillin, and glutamine. The studies reported here were carried out on cell strain 1146, derived from a 3-month old infant, obtained between the fifth and eighth passages. However, similar results were obtained with several other strains derived from normal human skin ranging in age from the 3rd month of gestation to the adult.

Confluent monolayer cultures were labeled approximately 1 week after passage with 20 μCi each of [U-^{14}C]proline, [U-^{14}C]lysine, and [U-^{14}C]glycine in 10 ml of Dulbecco-Vogt medium lacking these amino acids but supplemented with ascorbic acid (100 μg/ml) and β-aminopropionitrile hydrochloride (50 μg/ml). The medium was harvested and 50 μg/ml of *p*-mercuribenzoate (PMB)[1] and phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) were added to inhibit proteolysis. Collagenous proteins were precipitated from the medium with ammonium sulfate (20% saturation) at 4° overnight. The cell layer was suspended in 1 *M* NaCl, 100 m*M* EDTA, and 50 m*M* Tris-HCl (pH 7.4) containing 50 μg/ml each of $PhCH_2SO_2F$ and PMB. After 24 hr, soluble proteins were separated from insoluble materials by centrifugation and collagenous proteins were precipitated from the supernatant fluid with ammonium sulfate as indicated above. The precipitates from medium and cell layer were solubilized in 1% acetic acid and dialyzed exhaustively against 1% acetic acid prior to chromatography or electrophoresis.

In some studies larger quantities of the medium were collected from cells grown in roller bottles and stored at −20°. After thawing, the medium was concentrated with an Amicon hollow fiber concentrator, with pores excluding proteins with molecular weight below 50,000. Generally 20 l. of medium was reduced to a volume of 1–2 l. and at this time radioactive medium from labeled cultures was added to the concentrate. The collagenous proteins were then precipitated as described above.

The collagenous proteins were resolved by chromatography on DEAE-cellulose in 50 m*M* Tris-HCl–2 *M* urea (pH

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[1] Abbreviations used are: PMB, *p*-mercuribenzoate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

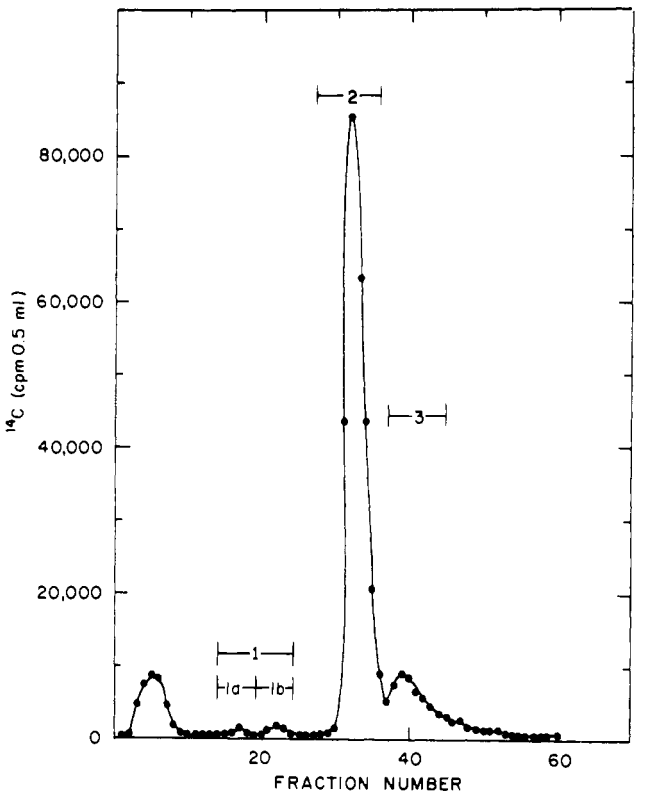

FIGURE 1: DEAE-cellulose chromatogram of the purified medium procollagen Chromatography was performed at 8°, with an elution volume of 600 ml and the gradient from 0 to 200 m*M* NaCl. Fractions of 10 ml were collected.

7.4) buffer with a linear gradient of 0–200 m*M* NaCl over a total volume of 600 ml at 8°. The fractions isolated by chromatography were dialyzed exhaustively against distilled water and lyophilized prior to further procedures.

Electrophoretic separations in SDS were carried out according to the procedures of Furthmayr and Timpl (1971). Collagen (20 μg) prepared from the skins of lathyritic rats was added to radioactive samples as carrier. After the completion of electrophoresis the gels were first fixed and stained in a solution of 0.2% Coomassie Blue, 50% methanol, and 5% acetic acid and then destained in 7% acetic acid–5% methanol. To localize radioactive bands, the gels were cut into thin sections (8 slices/cm of gel) which were incubated in 0.7 ml of a solubilizing solution (NCS, Amersham-Searle) for 2 hr at 50°. Subsequently, a toluene-based scintillation fluid (Spectrafluor, Amersham-Searle) was added and radioactivity in the sample measured by standard spectrometric methods.

To convert precursor forms to collagen-like molecules, the samples were incubated with pepsin at 15° for 4 hr (Layman et al., 1971). In some studies samples were digested with purified bacterial collagenase (Advanced Biofactures, Lynnwood, N.Y.) to distinguish which proteins contained collagenous portions (Peterkofsky, 1972).

To isolate the chains of the collagenous proteins, samples were first incubated for an hour in 8 *M* urea–10 m*M* Tris-HCl (pH 8.0) containing 100 m*M* β-mercaptoethanol at 50° and then dialyzed for an hour against 20 m*M* sodium acetate adjusted to pH 4.8. Components were resolved under denaturing conditions on CM-cellulose chromatography according to the method of Piez et al. (1963) in 0.02 *M* sodium acetate (pH 4.8) containing 4 *M* urea.

The identification of each type of procollagen was based on the profile of CNBr peptides. Prior to digestion with CNBr, disulfide bonds were reduced and the free sulfhydryls carboxymethylated (Monson and Bornstein, 1974). Cleavage with cyanogen bromide (CNBr) was performed after the addition of carrier collagen in 70% formic acid, for 5 hr at 30°. The resulting peptides were partially resolved by CM-cellulose chromatography in 20 m*M* sodium formate–30 m*M* NaCl (pH 3.8) at 45° using a linear salt gradient from 30 to 140 m*M* NaCl over a total volume of 100 ml on a column with dimensions of 9 × 120 mm at 15 ml/hr (Nicholls, unpublished data, 1974).

Type I collagen was prepared from lathyritic rat skins. Type II collagen was prepared from a rat chondrosarcoma as described by Smith et al. (1975). Type III collagen was prepared by pepsin treatment of skin from a stillborn infant (Chung and Miller, 1974). The pepsin-treated type III collagen was precipitated with 1.5 *M* NaCl–50 m*M* Tris-HCl (pH 7.4) and at this step contained 10–15% of type I collagen as judged by electrophoresis. Pepsin-treated type III collagen was eluted from CM-cellulose as described by Chung and Miller (1974). On subsequent electrophoresis a trimeric component was obtained from the CM-cellulose purified material which on reduction yielded only α1-type chains. The amino acid composition of material purified by chromatography was similar to those previously reported for type III collagen (Chung and Miller, 1974; Epstein, 1974).

Results

Genetically Distinct Collagens in the Medium. As much as 85% of the hydroxyproline containing proteins synthesized by human fibroblasts in culture are found in the medium. Ammonium sulfate (20% of saturation) precipitates native collagenous proteins while most other proteins and nonhelical collagen chains are eliminated at this step (Church et al., 1973). The proteins in the precipitate are resolved into three main radioactive fractions by chromatography on DEAE-cellulose (Figure 1). Peak 2 accounts for 75% of the radioactivity while peak 3 contains about 20%.

Previous studies have shown that α1 and α2 chains are obtained from peak 2 after limited cleavage with pepsin (Smith et al., 1972). Further identification of the DEAE-cellulose fractions from the human cells was established by comparing the profiles of radioactive CNBr peptides with those peptides obtained from type I, type II, and type III collagens. The profile of radioactive CNBr peptides from peak 2 was identical with that of the carrier peptides of type I collagen by chromatography on CM-cellulose, except that less radioactivity than expected was recovered with the carrier α1(I)-CB6 (Figure 2). Because the elution position of α1(I)-CB6 on CM-cellulose chromatography is variable (Rauterberg and Kühn, 1971), the peptides were partially resolved by electrophoresis. The expected amount of radioactive α1-CB6 was recovered on 7.5% SDS acrylamide gel electrophoresis (not shown).

The profile of the major CNBr peptides from peak 3 differed from the peptide profile of type I and type II collagens, but corresponded closely to those of type III collagen (Figures 3 and 4). On SDS acrylamide gel electrophoresis, type III collagen had an identifying peptide not present in type I collagen with a molecular weight of approximately 10,000, and an additional disulfide-linked peptide of molecular weight of approximately 70,000 which reduced to a peptide with a molecular weight of approximately 23,000. These studies clearly indicate that peak 2 contains protein

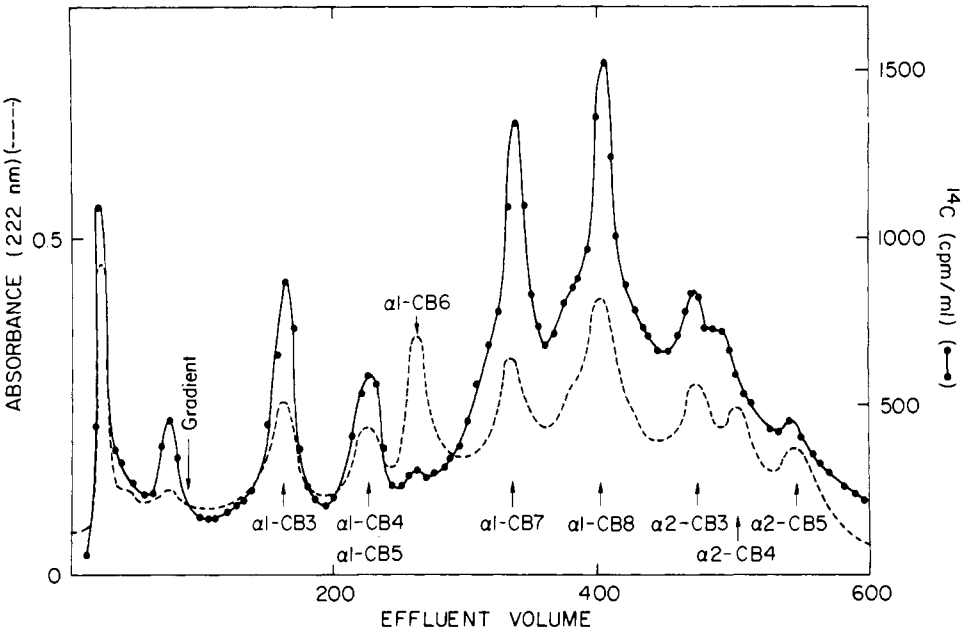

FIGURE 2: CM-cellulose chromatography at 40° of the radioactive CNBr peptides from the medium peak 2 and carrier type I collagen. The elution volume was 600 ml, with the salt gradient from 20 m*M* NaCl to 150 m*M* NaCl. Fractions of 4 ml were collected.

related to type I collagen and that peak 3 contains protein related to type III collagen.

Precursor Chains of Type I Collagen. The collagenous proteins in the cell culture medium have previously been resolved into several bands by SDS acrylamide gel electrophoresis (Goldberg et al., 1972). To relate these bands to specific collagens we first isolated the type I and III collagenous proteins in native form by DEAE-cellulose chromatography, then separated the denatured chains on CM-cellulose and finally sized each chain by SDS gel electrophoresis. Both radioactive procollagens and chemical quantities of procollagen from the medium of roller bottle cultures were used in these procedures. Approximately 10 mg of purified collagenous proteins could be obtained from 2 l. of medium. The procollagens from roller cultures had the same chromatographic and electrophoretic properties as the radioactive procollagen prepared from smaller culture flasks.

Two high molecular weight bands were obtained from denatured peak 2 on electrophoresis; however, following reduction four bands are present (Figure 5). After limited cleavage with pepsin the protein in peak 2 comigrated with $\alpha 1$ and $\alpha 2$ chains, in the expected 2:1 ratio, independent of reduction (Figure 5). No radioactivity was recovered in the high molecular weight region or α-chain region following incubation with purified bacterial collagenase. These results indicate that all four bands are collagenous and are precursor forms of $\alpha 1$(I) or $\alpha 2$. When chains were prepared by CM-cellulose chromatography from the protein in peak 2 after reduction and denaturation, three radioactive peaks were resolved, two of which eluted in the $\alpha 1$ region and one eluted near carrier $\alpha 2$ (Figure 6). The first peak eluting just prior to $\alpha 1$ migrated on electrophoresis with an estimated molecular weight of 120,000 and was designated p$\alpha 1$(I) (see Martin et al. (1975) for a discussion of this nomenclature). The second radioactive fraction from CM-cellulose (eluting with carrier $\alpha 1$) contained two components with molecular weights estimated to be 140,000 and 110,000. The higher molecular weight band was designated pro$\alpha 1$(I). The labeled peak that eluted from CM-cellulose

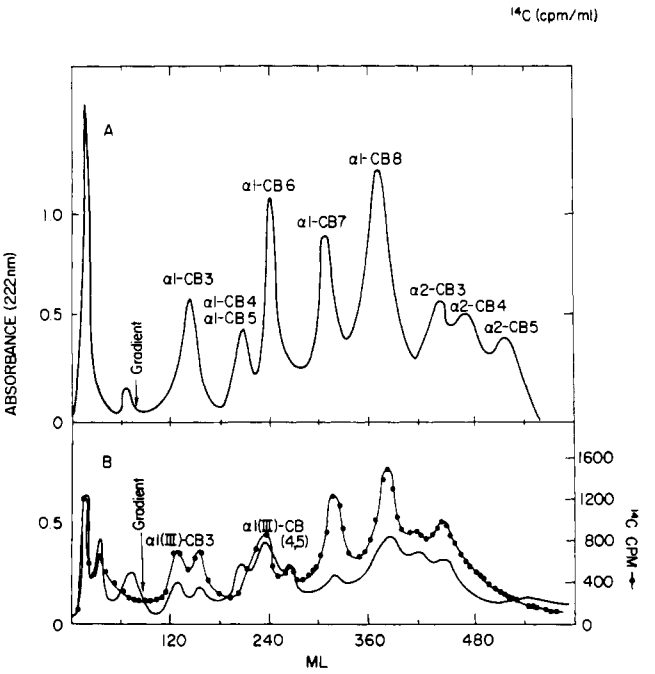

FIGURE 3: CM-cellulose chromatography of (A) type I collagen CNBr peptides from rat skin and (B) pepsin solubilized type III collagen CNBr peptides from human fetal skin and radioactive medium peak 3 CNBr peptides.

in the $\alpha 2$ region, on subsequent electrophoresis, was found to contain two components with molecular weights estimated (with $\alpha 2$ chain components as standards) to be 140,000 and 120,000. These were designated pro$\alpha 2$ and p$\alpha 2$, respectively.

Precursor Chains of Type III Collagen. The precursors of type III collagen generated only a single high molecular weight band on SDS gel electrophoresis prior to reduction, and two bands following reduction, which comigrated with pro$\alpha 1$(I) and p$\alpha 1$(I) (Figure 7a). In some samples a third band which coelectrophoresed with $\alpha 1$(I) was also present. After pepsin treatment, most of the radioactive protein mi-

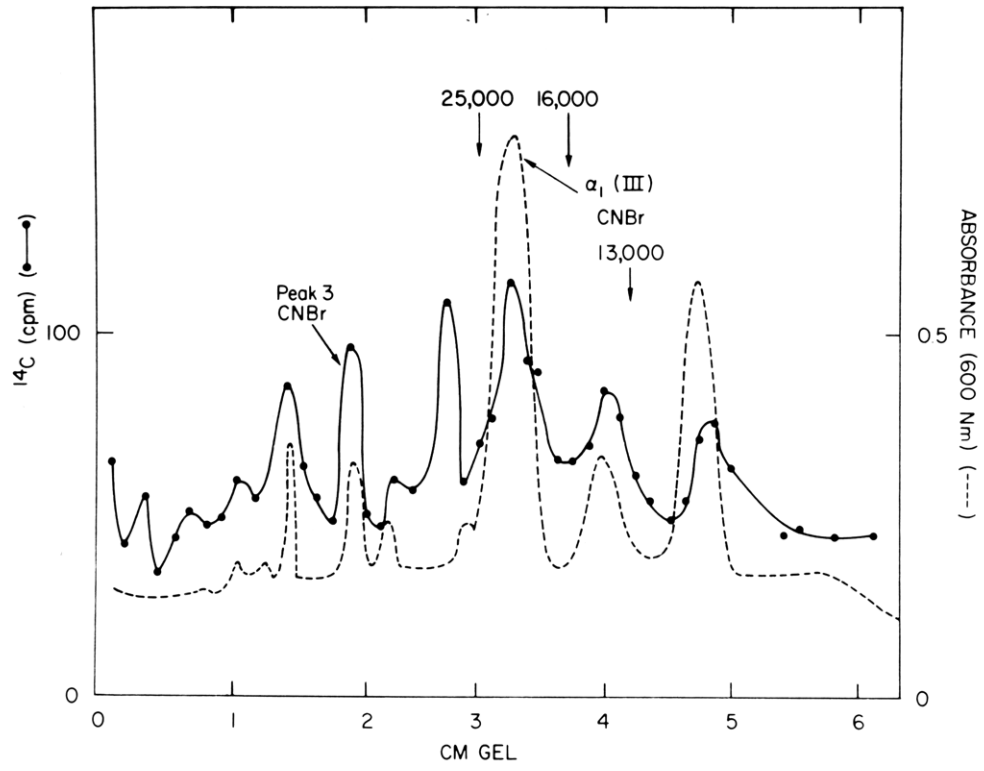

FIGURE 4: 7.5% SDS gel electrophoresis of radioactive medium peak 3 and pepsin solubilized human type III collagen, after reduction with β-mercaptoethanol.

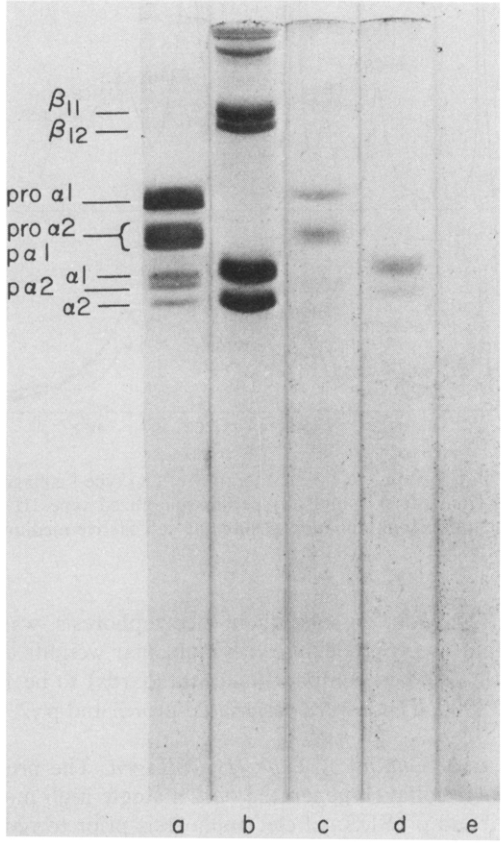

FIGURE 5: 5% SDS gel electrophoresis of (a) rat skin type I procollagen reduced with β-mercaptoethanol, (b) type I collagen standard, (c) medium peak 2 reduced, (d) medium peak 2, pepsin treated, (e) medium peak 2 collagenase treated.

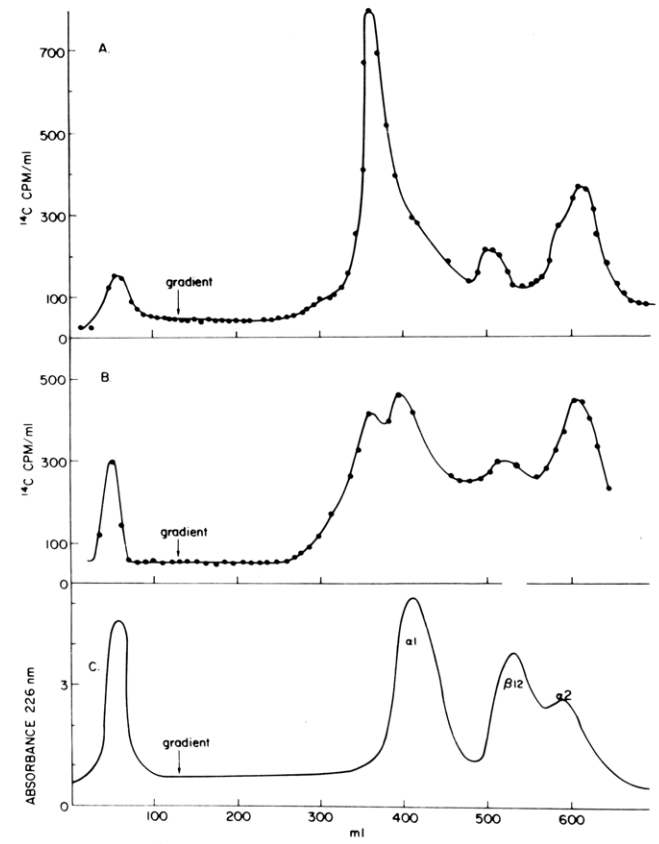

FIGURE 6: CM-cellulose chromatography at 40° after reduction and denaturation of (A) cell layer peak 2, (B) medium peak 2, and (C) lathyritic rat skin collagen. The elution volume was 800 ml, the gradient from 0 to 95 m*M*, and the fractions, 10 ml.

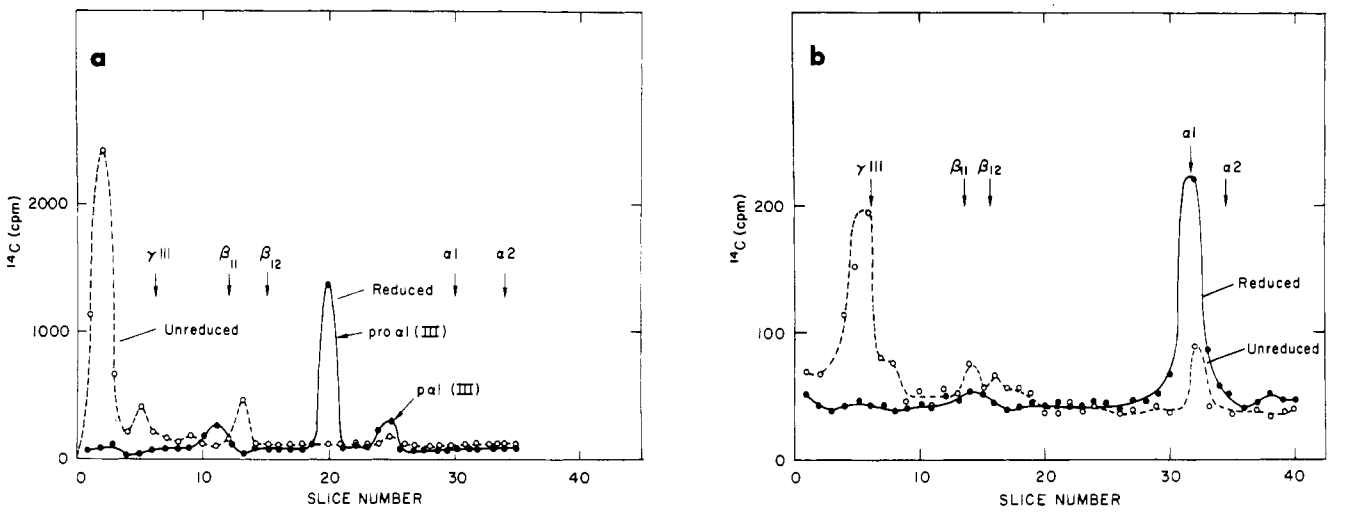

FIGURE 7: (a) 5% SDS gel electrophoresis of the radioactive material in medium peak 3 before and after reduction; (b) 5% SDS gel electrophoresis of the radioactive material in medium peak 3 after pepsin treatment, before and after reduction with β-mercaptoethanol.

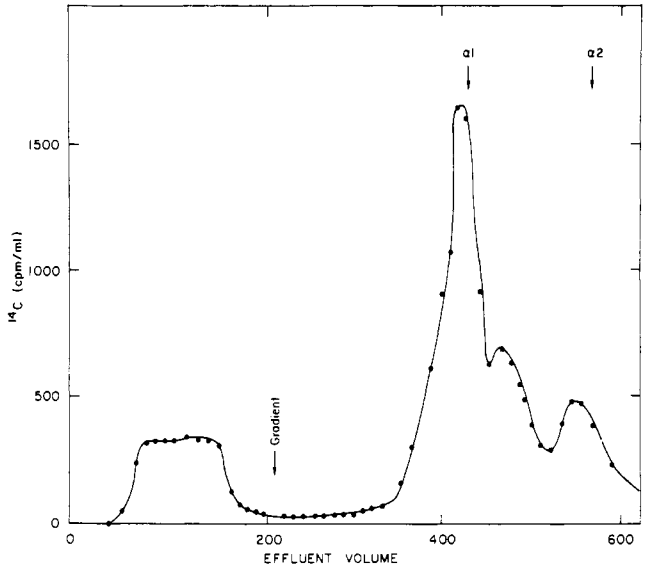

FIGURE 8: CM-cellulose chromatography after reduction and denaturation of the radioactivity material in medium peak 3.

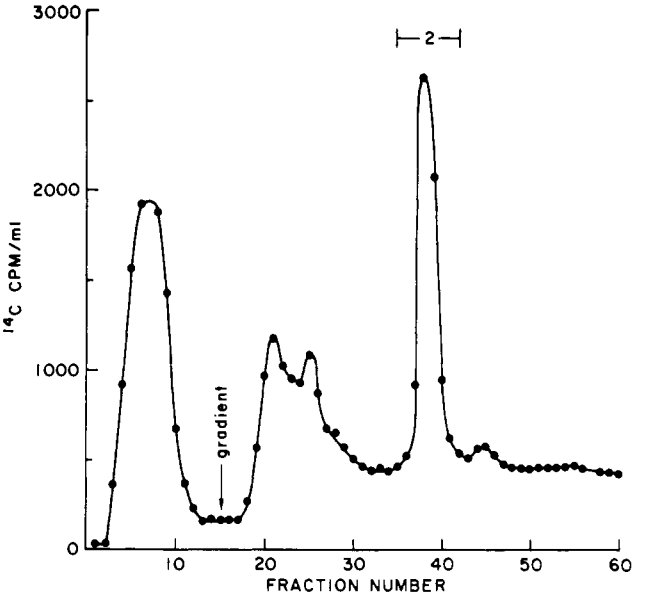

FIGURE 9: DEAE-cellulose chromatography of the purified collagenous proteins extracted from the cell layer. Conditions were the same as Figure 1.

grated near the origin; however, following reduction radioactivity was found in a single band comigrating with marker α1 chains (Figure 7b). Thus, peak 3 contains two precursor forms, proα1(III) and pαI(III), with estimated molecular weight of 140,000 and 120,000, respectively.

The chains of type III procollagen were also separated by CM-cellulose chromatography, after reduction and denaturation. Three proteins were separated, one major radioactive peak eluting with α1, and two smaller peaks eluting later (Figure 8). The smaller precursor chain, pα1(III), eluted first followed by proα1(III) and α1(III).

Precursor Chains of Type I Collagen in the Cell Layer. The collagenous proteins extracted from the cell layer were partially purified by ammonium sulfate precipitation and then chromatographed on DEAE-cellulose (Figure 9). The radioactivity applied eluted at the front and in additional peaks corresponding to peaks 1 and 2 from the medium. Little radioactivity was recovered in the region of peak 3. The CNBr peptide profile from the material in peak 2 was similar to that obtained with authentic type I collagen. When electrophoresed without reduction both high molecular weight bands (larger than 300,000) and bands which comigrated with pα1(I) and pα2 were resolved. Electrophoresis of reduced samples demonstrated only two bands of radioactivity comparable to pα1(I) and pα2 (with estimated molecular weight 120,000). Chromatography on CM-cellulose after reduction and denaturation resolved two major radioactive peaks (Figure 6A); the one preceding α1(I) migrated with pα1(I) on electrophoresis while the second peak eluting with carrier α2 migrated with pα2 on electrophoresis. These results indicate that this protein is type I p-collagen.

Discussion

Previous studies on the collagenous proteins synthesized by cultured human fibroblasts indicated that precursor forms of collagen (procollagen) accumulated in the medium. The major component of the medium collagenous proteins was identified as type I procollagen (Smith et al., 1972). A second component was shown to give rise to only

α1 type chains after limited proteolytic digestion (Church et al., 1973). Presumably, these two components represented different gene products although this was not shown.

In this study these collagenous proteins were isolated and the peptides, produced by digestion with CNBr, were compared with the peptides from authentic genetically distinct collagens. Little attention was paid to DEAE-cellulose peak 1 since the amount of material in this fraction was small and probably includes material degraded during isolation. Material in peak 2 was identified as type I collagen. Peptides attributable to both α1(I) and α2 chains were separated from the CNBr digest. Peak 3 was identified as a type III procollagen by CNBr derived peptides α1(III)-CB(3) and α1(III)-CB4,5 which separate from the homologous α1(I) chain peptides, and by the absence of α2 CNBr peptides (Miller et al., 1971; Chung et al., 1974). In addition, CNBr digests of α1(III) from the human contain a peptide of approximately 113 residues, not found in digests of α1(I) (Epstein, 1974). Epstein has used this peptide to estimate the proportion of type I to type III collagen in digests of skin, aorta, intestine, and other tissues. A peptide of this size is resolved from CNBr digests of peak 3 by electrophoresis.

As judged by the profiles of radioactive peptides produced by CNBr cleavage, peak 2 contains primarily type I collagenous protein and peak 3 primarily type III collagenous protein. This indicates that as a first approximation DEAE-cellulose chromatography can be used to measure precursors of type I and III collagens in cell culture and perhaps other systems. Also, the electrophoretic separation of CNBr peptides should prove useful for estimating tissue contents of genetically distinct collagens since little tissue is required. This procedure proved effective in recovering α1-CB6 which was recovered in low yield from CNBr digests of type I procollagen by CM-cellulose chromatography.

Subsequent efforts were directed toward identifying the polypeptide chains in these proteins. The chains in peak 2 protein were resolved by chromatography on CM-cellulose after denaturation and reduction. Two chains (140,000 and 120,000) chromatographed in the region of carrier α1(I) and two chains of comparable size with α2. Previous studies on the precursors of collagen (summarized by Martin et al., 1975) indicates that procollagen contains chains with molecular weights of 140,000. A shortened form p-collagen has been encountered in several systems and has chains of 110,000–120,000. Judged by their chromatographic properties and size, we suggest that the most acidic component eluted from CM-cellulose is pα1(I) and the chain chromatographing with carrier α1(I) is proα1(I). We suggest that the chains chromatographing with carrier α2 are proα2 and pα2. Both chain types are observed in the medium protein. As judged by electrophoresis in SDS, the proα forms are predominant in the medium while the pα chains are the exclusive form of precursor extracted from the cell layer.

Analogous chains were identified in the type III procollagen preparation. The largest chain, proα1(III), electrophoresed with proα1(I) and eluted with α1(I) on CM-cellulose chromatography. A chain tentatively identified as α1(III) elutes with α2. Similar chromatographic properties have been noted for the α1(III) chain from both rat and human skin (Byers et al., 1974; Chung and Miller, 1974).

As previously stated, the undegraded precursor of collagen is procollagen composed of three proα chains. While direct evidence is lacking it is possible that p-collagen composed of shortened proα chains is an intermediate in the conversion of procollagen to collagen suggesting a multistep conversion process. Both proα and pα chains are observed in organ culture, cell culture, and intact tissues. p-Collagen appears to be the major precursor form that accumulates in animals with defective conversion [the dermatosparaxic calves and patient having the type 7 form of the Ehlers-Danlos syndrome (Lenaers et al. 1971; Lichtenstein et al., 1973)]. Analogous chains are observed with both types I and III procollagens. Furthermore, the physical properties of these proteins differ since procollagen is soluble under physiological conditions while p-collagen is sparingly soluble and will precipitate. These observations are consistent with p-collagen being a physiological intermediate.

References

Burgeson, R. E., Wyke, A. W., and Fessler, J. H. (1972), *Biochem. Biophys. Res. Commun. 48,* 892.

Byers, P. H., McKenney, K. H., Lichtenstein, J. R., and Martin, G. R. (1974), *Biochemistry 13,* 5243.

Chung, E., Keele, E. M., and Miller, E. J. (1974), *Biochemistry 13,* 3459.

Chung, E., and Miller, E. J. (1974), *Science 183,* 1200.

Church, R. L., Tanzer, M. L., and Lapiere, C. M. (1973), *Nature New Biol. 244,* 188.

Epstein, E. H., Jr. (1974), *J. Biol. Chem. 249,* 3225.

Furthmayr, H., and Timpl, R. (1971), *Anal. Biochem. 41,* 510.

Goldberg, B., Epstein, E. H., Jr., Sherr, C. J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3655.

Layman, D. L., McGoodwin, E. B., and Martin, G. R. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 454.

Lenaers, A., Ansay, M., Nusgens, B. V., and Lapiere, C. M. (1971), *Eur. J. Biochem. 23,* 533.

Lichtenstein, J. R., Martin, G. R., Kohn, L. D., Byers, P. H., and McKusick, V. A. (1973), *Science 182,* 298.

Martin, G. R., Byers, P. H., and Piez, K. A. (1975), *Adv. Enzymol. Relat. Areas Mol. Biol.* (in press).

Miller, E. J., Epstein, E. H., Jr., and Piez, K. A. (1971), *Biochem. Biophys. Res. Commun. 42,* 1024.

Monson, J. M., and Bornstein, P. (1974), *Proc. Natl. Acad. Sci. U.S.A. 70,* 352.

Peterkofsky, B. (1972), *Arch. Biochem. Biophys. 152,* 318.

Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963), *Biochemistry 2,* 58.

Rauterberg, J., and Kühn, K. (1971), *Eur. J. Biochem. 19,* 398.

Smith, B. D., Byers, P. H., and Martin, G. R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3260.

Smith, B. D., Martin, G. R., Miller, E. J., Dorfman, A., and Swarm, R. (1975), *Arch. Biochem. Biophys. 166,* 181.