Biochemical Characterization of Collagens Synthesized by Fibroblasts Derived from Normal and Diseased Human Gingiva*

A. Sampath Narayanan and Roy C. Page

From the Department of Pathology and the Center for Research in Oral Biology, University of Washington, Seattle, Washington 98195

Fibroblasts obtained from healthy and diseased human gingiva were labeled with radioactive amino acids and the collagenous proteins synthesized were studied. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of untreated, reduced, and pepsin-treated proteins of the medium and cell extract showed that the collagenous proteins synthesized by these cells exist in the precursor form. Type I collagen was the chief constituent. In addition, cells from normal tissue synthesized type III collagen in amounts varying from 5 to 30%. Type III collagen was not detected in the cultures of fibroblasts from diseased tissue; however, an additional collagen fractionated between 2.5 to 5.0 M NaCl and accounted for 22 to 29% of the total. This collagen had an α1/α2 ratio of 8.6 and hydroxylsine/lysine ratio and cyanogen bromide peptide pattern were similar to that of α1[I]. It is concluded that the fibroblasts derived from diseased gingiva synthesize a collagen of composition (α1I)3, probably of type I.

Collagen is an essential component of all connective tissues. It is composed of three α chains of 1,050 amino acids each, bound into a helix of polyproline II type (1). Collagen is synthesized in the form of a precursor known as procollagen which is converted extracellularly to collagen by limited proteolysis (2). At least four different collagen types have been described. Type I collagen consists of two α1 chains and one α2 chain and is present in all connective tissues except cartilage (3). Type II, III, and IV molecules are composed of three α1 chains and these are present in cartilage, fetal tissues, and basement lamina, respectively (4-6).

Collagen is the principal component of human gingiva, and its most important structural component (7). The collagen fibers of this tissue exhibit an exquisite degree and pattern of organization (8) and serve the unique function of maintaining the organic union between the soft tissue and the calcified tooth surface. The gingival collagen seems to be unusual in that it turns over at a very high rate even in the adult, at least in subhuman primates (9). At a very early stage of inflammatory gingival and periodontal disease, the architecture of the gingival collagen fiber bundles is disrupted and almost 70% of the total collagen is lost (10, 11). As the disease progresses, this loss persists in some areas, while in the adjacent areas fibrosis occurs. These alterations are a cardinal feature of periodontal disease as well as of numerous other chronic inflammatory lesions.

The biochemical characteristics of collagen making up the normal marginal gingiva have not been defined and the nature of the collagenous alteration accompanying inflammatory disease remains poorly understood. We have analyzed the biochemical nature of the collagenous material synthesized in vitro by fibroblasts obtained from normal human gingiva and cells from the gingiva of an individual with severe chronic periodontitis.

EXPERIMENTAL PROCEDURE

Materials

Type I collagen of rat and human fetal skin was prepared by the method of Piez et al. (12) and type II collagen from articular cartilage of aborted human fetuses by the method of Miller (13). Human type III collagen was a gift of Dr. E. J. Miller; additional material was prepared from human fetal skin according to the method of Chung and Miller (14).

All radioactive amino acids and Aquasol were obtained from New England Nuclear, Boston, Mass. Pepsin (specific activity 3250 units/mg) was the product of Worthington Biochemical Corp., Freehold, N.J. β-Aminopropionitrile, iodoacetic acid, and mercaptoethanol were obtained from Calbiochem, La Jolla, Ca., and ion exchange celluloses from Whatman Biochemicals Ltd., Maidstone, Kent, United Kingdom. Chemicals for electrophoresis were the products of Bio-Rad, Richmond, Ca. All other chemicals not listed above were of analytical grade and purchased from Mallinckrodt, Baker, or Fisher Chemical.

Methods

Cell Culture—Fibroblasts were obtained from biopsies of the interproximal gingival papilla between the maxillary premolars of an individual with clinically and radiographically normal periodontal tissues who had undergone dental prophylaxis and 3 weeks of intensive oral hygiene, and from a comparable site in an individual with severe chronic periodontitis with extensive bone loss. Using standard techniques (15), fibroblasts were grown and maintained in 75-cm2 plastic culture flasks in Dulbecco-Vogt medium. Only cells between 5 and 15 doublings were used in the experiments described. Confluent monolayer cultures were labeled 10 days after passage with 5 μCi/ml of

* This work was supported by National Institutes of Health Grants DE-03301, DE-02600, and HL-03174.
[G-3H]proline or [3 H]glycine or both, or 2 μCi/ml of [U-14C]glycine in Dulbecco-Vogt medium lacking these amino acids and serum but containing 50 μg/ml each of ascorbic acid and β-aminopropionitrile. The latter compound specifically inhibits lysyl oxidase and prevents the newly synthesized collagen from becoming cross-linked (16).

Cultures were incubated for 24 h at 37°C in an atmosphere of 5% CO2 and 96% air. The medium was harvested and the cell layer extracted twice by stirring overnight in 5 ml/flask of 0.5 M acetic acid. The insoluble residue was removed by centrifugation and the two extracts combined. Unincorporated radioactivity was eliminated by dialysis, and the medium, cell extract, and insoluble residue were analyzed separately.

Limited Pepsin Digestion—The cell extract in 0.5 M acetic acid or medium and other samples previously dialyzed against 0.5 M acetic acid, were treated with 100 μg/ml of pepsin for 6 h at 15°C (15). The pH was then adjusted to 7.5 to 8.0 with NaOH to inactivate the pepsin.

Medium and other samples previously dialyzed against 0.5 M acetic acid, were treated with 100 μg/ml of pepsin for 6 h at 15°C (15). The pH was then adjusted to 7.5 to 8.0 with NaOH to inactivate the pepsin.

CM-cellulose Chromatography—Separation of the various collagen α chains was done on a CM-cellulose column (0.9 x 9.0 cm) at 42°C as described by Piesz et al. (12) using 0.02 M sodium acetate buffer, pH 4.5, containing 1 M urea with a NaCl gradient of 0 to 110 mM in a total volume of 200 ml of buffer. The column was standardized with human type I, II, and III collagens. During the chromatography, the samples contained 5 mg of lathyritic rat skin collagen as internal marker and carrier. Cyanogen bromide peptides were separated on column (0.9 x 19 cm) at 42°C with NaCl gradient of 0.03 to 0.17 M in 200 ml of 0.02 M sodium formate buffer, pH 3.8 (17).

Reduction and Alkylation—Samples were reduced by stirring at room temperature in 0.05 M Tris-HCl buffer, pH 7.4, containing 8 M urea and 50 mM mercaptoethanol (18). After 6 h they were cooled in ice and stirred for 30 min with 100 mM iodoacetamide. The samples were then dialyzed to remove urea and lyophilized.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—α and pro-α chains of collagen were separated in 5% gels by the method of Weber and Osborn (19). The electrophoresis was done in the absence of mercaptoethanol except when specified. Each gel contained 50 μg of lathyritic rat skin collagen as internal marker and carrier. Molecular weights were calculated from the migration of collagen chains. Resolution of cyanogen bromide peptides was achieved in 7.5% gels by comparing to 145,000 and 120,000 corresponding to pro-α1 and pro-α2 collagen chains, respectively (Fig. 1, B and E). After pepsin digestion the material migrated in the position of (~1 100,000) pro-collagen chains was done on a CM-cellulose column (0.9 x 9.0 cm) at 42°C as described by Piesz et al. (12) using 0.02 M sodium acetate buffer, pH 4.5, containing 1 M urea with a NaCl gradient of 0 to 110 mM in a total volume of 200 ml of buffer. The column was standardized with human type I, II, and III collagens. During the chromatography, the samples contained 5 mg of lathyritic rat skin collagen as internal marker and carrier. Cyanogen bromide peptides were separated on column (0.9 x 19 cm) at 42°C with NaCl gradient of 0.03 to 0.17 M in 200 ml of 0.02 M sodium formate buffer, pH 3.8 (17).

Resolution of cyanogen bromide peptides was achieved in 7.5% gels by comparing to 145,000 and 120,000 corresponding to pro-α1 and pro-α2 collagen chains, respectively (Fig. 1, B and E). After pepsin digestion the material migrated in the position of (~1 100,000) pro-collagen chains was done on a CM-cellulose column (0.9 x 9.0 cm) at 42°C as described by Piesz et al. (12) using 0.02 M sodium acetate buffer, pH 4.5, containing 1 M urea with a NaCl gradient of 0 to 110 mM in a total volume of 200 ml of buffer. The column was standardized with human type I, II, and III collagens. During the chromatography, the samples contained 5 mg of lathyritic rat skin collagen as internal marker and carrier. Cyanogen bromide peptides were separated on column (0.9 x 19 cm) at 42°C with NaCl gradient of 0.03 to 0.17 M in 200 ml of 0.02 M sodium formate buffer, pH 3.8 (17).

Salt Fractionation—The various collagen types were separated by differential salt precipitation by the methods described by Chung and Miller (14) and Twedtal et al. (21). The sample was digested with pepsin and neutralized as described before. Then, 5 mg of lathyritic rat skin collagen carrier was added followed by solid NaCl to 5M. The mixture was allowed to stand overnight at 4°C and the collagen precipitate was collected by centrifugation at 27,000 x g for 15 min. Both the pellet and the supernatant were retained. The pellet was washed once with 5 M NaCl in 0.05 M Tris-HCl buffer, pH 7.4, and extracted sequentially in Tris buffer containing 2.5 M NaCl and 1.5 M NaCl, respectively. In this procedure, type III collagen remains in the 1.5 M NaCl pellet (14), type I collagen appears in the 1.5 M salt extract (14), and type II molecules are located in the 2.5 M NaCl extract (21). The fractions were desalted by dialysis, and lyophilized prior to CM-cellulose chromatography.

Cyanogen Bromide Digestion—The radioactive proteins to be digested contained 5 to 10 mg of purified human α1(I) as carrier. A 5-fold excess (w/w) of cyanogen bromide was added and the collagen digested for 4 h at 30°C (5). Human type II collagen was also digested simultaneously.

Amino Acid Analysis—Samples were hydrolyzed for 24 h in constant boiling HCl at 108°C and the radioactive amino acids separated on a Beckman 120C amino acid analyzer equipped with a stream-splitting device. Fractions were collected and the radioactivity in fractions measured by counting aliquots in Aquasol.

RESULTS

Characterization of Collagens Synthesized by Fibroblasts from Normal Gingiva—Incorporation of the radioactive

The mobilities of α1 and α2 chains of collagen and cyanogen bromide peptides were the same in the presence or absence of mercaptoethanol, therefore the electrophoresis was usually done in the absence of mercaptoethanol.

TABLE I

<table>
<thead>
<tr>
<th>Collagens Synthesized by Gingiva Fibroblasts in Vitro</th>
<th>5465</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of collagen and noncollagenous proteins by human gingiva fibroblasts</td>
<td>5465</td>
</tr>
<tr>
<td>The cell residue and portions of culture medium and cell extract were dialyzed extensively to remove unincorporated proline, lyophilized, and hydrolyzed. The radioactivity in hydroxyproline and proline were determined as described under “Methods,” and the counts per culture reported.</td>
<td>5465</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fibroblast source</th>
<th>Fraction</th>
<th>Hyp</th>
<th>Pro</th>
<th>Hyp/Pro</th>
<th>Per cent of total collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue</td>
<td>Medium</td>
<td>31</td>
<td>97</td>
<td>0.32</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Cell extract</td>
<td>16</td>
<td>148</td>
<td>0.11</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Cell residue</td>
<td>3</td>
<td>121</td>
<td>0.02</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50</td>
<td>366</td>
<td>0.14</td>
<td>100</td>
</tr>
</tbody>
</table>

| Diseased tissue   | Medium   | 50  | 222 | 0.23    | 86                       |
|                   | Cell extract | 6   | 101 | 0.06    | 10                       |
|                   | Cell residue | 2   | 96  | 0.02    | 3                        |
|                   | Total      | 58  | 419 | 0.14    | 100                      |

proline in collagenous and noncollagenous proteins during a 24-h period by cultures of confluent fibroblasts from normal human gingiva is shown in Table I. While the major portion of the radioactive protein, as manifested by the counts in proline, was associated with the cells, collagens proteins, as evidenced by the radioactivity present in hydroxyproline, were found predominantly in the culture medium. The distribution of hydroxyproline in the medium, cell extract, and cell residue was 63, 32, and 5%, respectively. The culture medium and the cell extract were subjected to sodium dodecyl sulfate-polycrylamide gel electrophoresis in order to establish the identity of the collagenous material and to evaluate the extent of cross-linking. The results of these experiments are presented in Fig. 1. In the untreated preparations of both culture medium and cell extract, the radioactive proteins remained near the origin (Fig. 1, A and D), indicating a molecular weight greater than that of collagen. Following reduction and alkylation two peaks appeared with approximate molecular weights of 140,000 to 145,000 and 120,000 to 123,000 corresponding to pro-α1 and pro-α2 collagen chains, respectively (Fig. 1, B and E). After pepsin digestion the material migrated in the position of α1 and α2 chains of collagen (Fig. 1, C and F). Since procollagen molecules behave in this manner (2, 22), these observations show that the collagenous proteins synthesized by the normal cells under these conditions exist as procollagen.

The medium and cell extract proteins were characterized further as described below. Because a greater portion of radioactive collagen was present in the medium, and since essentially the same results were obtained for both preparations, only data from the medium are presented.

An attempt was made to separate the collagen chains by CM-cellulose chromatography using standard methods (12). However, a major portion of the radioactivity did not elute from the column, and almost no radioactive α chains were obtained (Fig. 2A); reduced and alkylated medium behaved similarly (Fig. 2B). However, samples previously digested with pepsin contained discrete peaks in the position of α1 and α2.
Collagens Synthesized by Gingiva Fibroblasts in Vitro

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of medium proteins and 0.5 M acetic acid extract of cultures of normal human gingival fibroblasts labeled with [3H]proline. Equal aliquots of medium or cell extract were treated and subjected to electrophoresis. The left panel (A, B, C) represents the data for medium and the right panel (D, E, F) represents cell extract. A and D, untreated; B and E, after reduction and alkylation; C and F, after limited digestion with pepsin. The mobilities of γ, β, and α1 chains of rat skin collagen, which were used as internal markers, are indicated. Radioactivity of each 1-mm slice is shown.

FIG. 2. CM-cellulose chromatography of medium collagen produced by fibroblasts from normal gingiva and labeled with [3H]proline and [2-3H]glycine. A, untreated; 297.1 x 10^3 cpm were loaded and 5.6 x 10^2 (Fig. 2C), indicating that the collagens synthesized by the normal gingiva cells exist in the precursor form.

The material synthesized by the gingival cells was eluted from a DEAE-cellulose column using buffer containing 2 M urea under conditions expected to separate Type I and III pro-collagens (18). However, only 2 to 3% of the material loaded was recovered. The addition of 6 M urea to the buffer system permitted recovery of about 40% of the material, but the separation of procollagens was poor. Therefore, the material was subjected to pepsin digestion and salt fractionation by the methods of Trelstad et al. (21) and Chung and Miller (14), followed by characterization using CM-cellulose chromatography. In a typical experiment, the 5.0 M NaCl supernatant contained 47% of the total radioactivity but only negligible amounts of collagenous protein (Table II, Fig. 3A). The 2.5 to 5.0 M NaCl fraction, which is expected to contain type II molecules if present, contained only 4% of the total collagenous protein and on CM-cellulose chromatography revealed an α1/α2 chain ratio of 2.8 (Table II, Fig. 3B); thus this fraction appeared to be type I collagen. The 1.5 to 2.5 M NaCl fraction, which is expected to contain type I molecules, had 87% of the total collagen, eluted from CM-cellulose as α1 and α2 chains with a ratio of 2.6 (Fig. 3C), indicating that it is type I collagen. Approximately 8% of the total collagenous material appeared in the 1.5 M NaCl fraction, which is expected to contain type III collagen. On CM-cellulose chromatography, this fraction revealed a peak eluting in the region of type III chains and in the experiment presented this peak accounted for 5% of the total collagen (Table II, Fig. 3D). To confirm the identity of the putative type III chains, the fractions under this peak were pooled, desalted, and examined electrophoretically with and without reduction and alkylation. Since type III molecules

FIG. 2. CM-cellulose chromatography of medium collagen produced by fibroblasts from normal gingiva and labeled with [3H]proline and [2-3H]glycine. A, untreated; 297.1 x 10^3 cpm were loaded and 5.6 x 10^2 cpm were recovered in the fractions. B, reduced and alkylated; 74.6 x 10^2 cpm were loaded. C, digested with pepsin; 158 x 10^2 cpm were loaded. Fractions of 6.9 ml were collected and the radioactivity of 1.0 ml portions is shown. In C the radioactivity of α1 [I], α1 [III], and α2 peaks were 3080, 160, and 1350 cpm, respectively (α1/α2 = 2.3). The top figure represents the ultraviolet scan of rat skin collagen; human α2 elutes slightly later and the elution of human α1, α1 [III], and α2 chains are indicated.

TABLE II

Distribution of radioactivity and collagen in various NaCl fractions of medium from fibroblasts of normal gingiva labeled with [3H]proline

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity, total proteins cpm x 10^-3</th>
<th>Per cent of total</th>
<th>Radioactivity, collagen cpm x 10^1</th>
<th>Per cent of total α1/α2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M NaCl pellet</td>
<td>18 8</td>
<td>Type I&lt;sup&gt;a&lt;/sup&gt; 15 3</td>
<td>1.5</td>
<td>α1/α2</td>
</tr>
<tr>
<td>1.5-2.5 M NaCl</td>
<td>91 41</td>
<td>Type III&lt;sup&gt;a&lt;/sup&gt; 391 87</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>2.5-5.0 M NaCl</td>
<td>8 4</td>
<td></td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>5.0 M NaCl supernatant</td>
<td>105 47</td>
<td>Negligible</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Total radioactivity under α1 [I] plus α2 peaks.

<sup>b</sup>Total radioactivity under α1 [III] peak.

The term "α1" has been used in reference to the α1 chains that will elute on CM-cellulose chromatography in the position of rat skin α1 [I]. This fraction might derive from type I (14), II (4), or IV (6) collagens, and therefore may be a mixture of more than one type. However, presence of α2 and α1/α2 ratio of 2.0 would indicate that it is derived from type I collagen. When necessary, we have indicated the type of collagen in parentheses.
had a disulfide bond within the helical portion of the molecule, they exhibit a molecular weight of about 300,000 prior to reduction and 100,000 after reduction (5, 14). As seen in Fig. 4, the unreduced material remained near origin with γ chains (Mγ = 300,000), whereas after reduction it migrated with the α1 chains (Mα1 = 100,000), thus confirming type III identity.

Collagen of Cell Residue of Fibroblasts from Normal Human Gingiva—As noted in Table I, about 5% of the total collagenous protein synthesized by the normal gingival fibroblasts remained in the cell residue after extraction with 0.5 N acetic acid. The residue was suspended in 0.5 N acetic acid and solubilized by overnight digestion at 4°C with pepsin (1 mg/ml) (6, 13). The solubilized material was subjected to salt precipitation and analysis by CM-cellulose chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only α1(I) and α2 chains, in a ratio of 2.0 to 2.5, were found, thereby indicating that only type I collagen is present in the cell residue. The small amount of radioactive collagen obtained precluded further characterization by cyanogen bromide digestion.

Characterization of Collagens Synthesized by Fibroblasts Derived from Diseased Gingiva—Morphologically, no differences were noted between the cells obtained from normal gingiva and those from the diseased tissue. Although cell counts were not done, the cells from the diseased tissue appeared to grow more slowly than those from the normal tissue and, under identical transfer conditions, more time was required for the former to become confluent. Cultures of the two types did not differ significantly in their capacity to synthesize protein or collagen (Table I), although more of the collagenous material synthesized by the cells from the abnormal tissue was present in the medium (Table I). Distribution of collagenous protein in the absence of β-aminopropionitrile was not evaluated.

Radioactive proteins synthesized by the cells from diseased gingiva remained near the origin when examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 5A). After reduction and alkylation, peaks of lower molecular weight appeared and these migrated in the region of pro-α1 (Mα1 = 145,000) and pro-α2 (Mα2 = 118,000) chains (Fig. 5B). Material subjected to limited pepsin digestion migrated in the region of α1 and α2 chains (Fig. 5C). The radioactive proteins of the cell extract behaved in the same manner (not shown). Thus, as in the case of normal cells, procollagen rather than collagen predominated in the cultures.

Attempts at elution of the untreated medium collagen from CM-cellulose columns were not successful; very little material was recovered under the α chain peaks (Fig. 6A). Recoveries were not improved by reduction and alkylation (Fig. 6B), but following limited pepsin digestion discrete peaks of α1 and α2 chains were present (Fig. 6C). The α1/α2 ratio ranged from 3.4 to 4.8, a value considerably higher than 2.0 expected for type I collagen, and indicating possible molecular heterogeneity. Therefore, the pepsin-digested material was subjected to salt fractionation and characterization (Table III, Fig. 7). The 1.5 M

When cells from one confluent flask were transferred to two flasks, the normal cells became confluent in 3 to 4 days, the abnormal cells in 5 to 7 days.

4 Reduction and alkylation yields considerably more pro-α2 than expected of type I collagen. This could be due to the presence of partially degraded pro-α1 chains along with pro-α2. The presence of low molecular weight peaks (see 4- to 5-cm portion of the gel in Fig. 5B) supports this possibility. Alternatively, the precursor of the unusual α1 chain described later may migrate along with pro-α2.
Sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern of medium from cells of diseased human gingiva labeled with $^{3}H$proline. Equal portions of medium were treated and applied to gels. A, untreated; B, reduced and alkylated; C, digested with pepsin. Radioactivity of each 2-mm slice is shown, thus resolution is not as good as in Fig. 1. The migration of collagen chains is indicated.

**FIG. 6.** CM-cellulose chromatography of medium collagen produced by cells of the diseased gingiva and labeled with $[^{3}H]$proline. Fractions of 6.9 ml were collected and 1.0-ml portions counted. The top figure shows the ultraviolet scan of rat skin collagen carrier. The elution of human $\alpha_1$, $\alpha_1[III]$, and $\alpha_2$ is shown. In this system $\alpha_1[IV]$ chains elute in the same position. A, untreated; 94.5 × 10⁴ cpm were loaded. B, reduced and alkylated; 73.6 × 10⁴ cpm were separated. C, after limited digestion with pepsin; 58.7 × 10⁴ cpm were loaded. The radioactivity under $\alpha_1$ and $\alpha_2$ peaks, respectively, were 1027 cpm (7086 cpm total) and 266 cpm (1835 cpm total). The $\alpha_1/\alpha_2$ ratio was 3.9.

**TABLE III**

Distribution of radioactivity and collagen in various NaCl fractions of medium from fibroblasts of diseased gingiva

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity of total proteins</th>
<th>Collagen radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm × 10⁴</td>
<td>Per cent of total</td>
</tr>
<tr>
<td>1.5 M NaCl pellet</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>1.5-2.5 M NaCl</td>
<td>58</td>
<td>26</td>
</tr>
<tr>
<td>2.5-5.0 M NaCl</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>5.0 M NaCl supernatant</td>
<td>77</td>
<td>61</td>
</tr>
</tbody>
</table>

NaCl fraction contained only 2% of the total collagen (Table III) and exhibited an $\alpha_1$ and $\alpha_2$ chain ratio of 2.5, typical of type I collagen; no type III molecules were detected (Fig. 7A). The 2.5 M NaCl fraction, containing 76% of the total collagen, was made up of $\alpha_1$ and $\alpha_2$ chains in a ratio of 9.5, indicating an identity of type I collagen (Table III, Fig. 7B). Twenty-two percent (22 to 29% in several experiments) of the total collagenous material remained in 2.5 to 5.0 M NaCl fraction (Table III). On CM-cellulose this fraction had both $\alpha_1$ and $\alpha_2$ chains, but the ratio was > 8.0 (4.4 to 8.6 in several experiments) (Fig. 7C).
Table III, indicating the presence of excess α1 chains. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that this fraction had a molecular weight of 100,000 (Fig. 8). The 5.0 M NaCl supernatant fraction had 57% of the total radioactivity but did not contain significant amounts of collagen (Table III, Fig. 7D).

In the CM-cellulose procedure employed here, α1[I], α1[II], and α1[IV] chains elute together (4, 6). Therefore, the high α1/α2 ratio of the collagen of the 2.5 to 5.0 M NaCl fraction (which is expected to contain type II collagen) shown in Fig. 7C would indicate the presence of these α chains. To verify the presence of these α chains the following experiment was done. Type II and IV chains differ markedly from type I chain in the extent to which lysine is hydroxylated. While 65 and 64% of the lysines are hydroxylated in α1[II] and α1[IV], respectively (6, 23), only about 15% of these are hydroxylated in α1[I]. Therefore, the cells were labeled with [14C]lysine and α1 chains prepared as described above. The radioactivity in lysine and hydroxylysine of these preparations was measured by amino acid analysis and liquid scintillation counting. As shown in Table IV, the extent of hydroxylation of lysine of the α1 chains from the 2.5 to 5.0 M NaCl fraction was not sufficiently high to indicate the presence of type II or type IV collagens.

The α1 chains from the 2.5 to 5.0 M NaCl and the 1.5 to 2.5 M NaCl fractions, illustrated in Figs. 7, C and B, were subjected to cyanogen bromide digestion and the peptide pattern was compared by CM-cellulose chromatography and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with those of α1[I] and α1[II] chains. As shown in Fig. 9, the CM-cellulose pattern of the unknown material closely resembled that of the standard human α1[I] chains and the α1[II] chain from the 1.5 to 2.5 M NaCl fraction. Furthermore, the marker peptide α1[II]CB10 (M, 31,000) (25) was not present in the unknown material. The same conclusions were reached from the electrophoretic experiments shown in Fig. 10.

**Collagen of Cell Residue of Fibroblasts from Diseased Gingiva**—The cell residue collagen accounted for only 3% of the total synthesized by cells from the diseased tissue. CM-cellulose chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins before and after limited pepsin digestion and reduction, and by DEAE-cellulose chromatography, very similar results have been obtained by others using

**DISCUSSION**

Fibroblasts obtained from human gingiva synthesize quantities of collagenous proteins sufficiently large to permit biochemical characterization. Approximately 60% of the collagenous material remains in the culture medium. Most of the material is procollagen as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and CM-cellulose chromatography of the proteins before and after limited pepsin digestion and reduction, and by DEAE-cellulose chromatography. Very similar results have been obtained by others using
Collagens Synthesized by Gingiva Fibroblasts in Vitro

The ability of the cells from diseased tissue to produce the unusual collagen and their apparent inability to make type III molecules persist in cultures through numerous cell generations. Thus these cells appear to be genetically different from those obtained from explants of normal gingiva. Mechanisms underlying such a change remain obscure, although several possibilities are apparent. We may have inadvertently detected a previously unknown genetic polymorphism of the collagen locus, although this seems unlikely because the unusual behavior is exhibited by cells from two patients and was not seen in those from two normal individuals. Inflamed gingiva contains a dense plasma cell and neutrophil infiltrate and there is considerable tissue destruction. Conditions of this type may modulate fibroblast synthetic activity, although a change of this type would not be expected to persist in cultures. The most likely mechanism to account for our observations appears to be one of cell selection. The experiments of Martin et al. (39) show that fibroblast populations may be heterogeneous. Chronically inflamed tissues contain toxic substances such as lymphokines and cellular hormones such as lymphocytes.
Collagens Synthesized by Gingiva Fibroblasts in Vitro

as the prostaglandins; these substances may create selective growth pressures leading to modulation in fibroblast populations.

REFERENCES
