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Dilantin Hyperplasia. Biochemical Studies On The Collagen And Noncollagenous Proteins Of Human Gingivae.

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DILANTIN HYPERPLASIA. BIOCHEMICAL STUDIES ON THE COLLAGEN AND NONCOLLAGENOUS PROTEINS OF HUMAN GINGIVAE.

BY

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Persons too numerous to mention have assisted me in this work and I would like to take this opportunity to express my appreciation to them. There is one person, however, who has made the greatest contribution and who is largely responsible for whatever I have achieved. To my wife, Ellen, I therefore gratefully dedicate this work.

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INTRODUCTION

Dilantin, or diphenylhydantoin (DPH), currently one of the most effective drugs for treating grand mal and other seizure disorders, often causes hyperplasia of the gingival tissues when used for prolonged periods. Though the hyperplasia appears to be a connective tissue reaction, the cause, incidence, and management of this problem remain highly controversial issues.

A scan of the current articles and reviews on hyperplasia showed that numerous clinical and laboratory studies have led to an almost equal number of different conclusions concerning the disease. Since the histological studies of the affected gingiva were more numerous than those of a biochemical nature, it seemed of value to examine the gingival collagen. The current wealth of information being reported on the biochemical aspects of connective tissue elements, particularly collagen, further suggested the timeliness for definitive studies on the character of the fibers found in gingivae and other periodontal structures.

REVIEW OF LITERATURE

The literature concerning DPH and its effect on gingival hyperplasia is so extensive that this review will be restricted to articles published since 1939. These selected articles illustrate the difficulty of arriving at reasonable solutions to the many problems that have been associated with the use of this drug. Members of the dental profession, in private practice and in research, have been particularly interested in epilepsy since 1939, when patients who were being treated for this disease with DPH subsequently developed gingival hyperplasia.

DPH is variously referred to as sodium diphenylhydantoin; Dilantin sodium; sodium diphenylhydantoinate; phenytoin sodium; sodium 5,5-diphenylhydantoinate; 5,5-diphenyl glycolurea; and Epanutin.

The convulsive disorders of epilepsy are grouped into four major categories: petit mal, grand mal, Jacksonian epilepsy, and psychomotor epilepsy (1). At one time epileptics with grand mal and psychomotor seizures were given bromide therapy. Reader (77) showed the barbiturate Luminal to be useful in controlling such seizures. The more recently discovered Dilantin controlled the seizures equally as well as the

other two drugs but did not cause the drowsiness and mental depression they induced.

Incidence of Gingival Hyperplasia Resulting from DPH

Experiments on cats (61) suggested that DPH might benefit the epileptic. Shortly after DPH was first used to treat humans, it was reported by Kimball (47) that the drug might cause gingival hyperplasia. Reader (77) and Angelopoulos and Goaz (2) reviewed a total of some 60 reports related to DPH research carried out between 1938 and 1972. In these investigations, the recorded incidence of hyperplasia ranged from 0-85% (Table I). The overall average was 37%. In their review, Angelopoulos and Goaz questioned the validity of including material from all the early reports for comparing the findings on the drug. They reasoned that it was not the primary purpose of some of the early studies to determine the degree of DPH gingival hyperplasia, but that the condition had only been an incidental finding. Furthermore, no standard method for collecting data was available. Therefore, these reviewers suggested that, for comparative purposes, the exclusion of all reports published before 1950 would narrow the range of incidence to between 32-85%, and would still indicate

the discrepancies among the findings of the different studies. Statistical analysis of the findings from the 1972 study of Angelopoulous and Goaz (2) revealed no relation between incidence of hyperplasia and age, sex, DPH dosage, or duration of treatment. However, the influence of good oral hygiene in maintaining normal gingiva during DPH treatment was clinically evident. In a study by Gardner, Gross, and Wynne (34), mentally retarded patients who were treated with DPH were grouped as spastics and nonspastics. Most of these patients exhibited some degree of gingival hyperplasia. It was noted that no correlation existed between frequency of tooth brushing and the degree of hyperplasia, and that all patients used improper brushing techniques. Ziskin, Stowe, and Zegarelli (101) reported that, in their study, the hyperplasia of patients disappeared after all teeth were extracted, but recurred, in some cases, when dentures were inserted.

Klar (49) observed some degree of gingival hyperplasia in 63% of 312 outpatients who were receiving DPH for seizure disorders. Hyperplasia was more prevalent among the patients who had received DPH longer than 2-3 years. Although the oral hygiene index appeared to correlate with the incidence of hyperplasia, this did not prove to be statistically significant.

In the investigations described, most patients had received DPH treatment prior to the studies; some had been treated for longer periods than others. Some workers felt that good oral hygiene could prevent gingival hyperplasia. Seeking to determine whether good oral hygiene could minimize gingival hyperplasia, Ciancio, Yaffe, and Catz (19) examined 12 outpatient children (who were subject to cerebral seizures) within 10 days after DPH therapy was started. The only patient in this study to develop gingival hyperplasia was one who did not practice good oral hygiene. It was thought that this 120-day study may have stimulated an oral awareness that caused the participants to practice better than usual oral hygiene, irrespective of group assignment.

Review of Clinical and Microscopic Features of DPH Gingival Hyperplasia

The gingival overgrowth resulting from the use of DPH has been referred to as hypertrophic gingivitis, inflammatory gingival enlargement, hypertrophy, hyperplasia, or a combination of the last two. Hypertrophy was the term that was first widely used to designate this disease, but, in the 1947 report of the Committee on Nomenclature of the American Academy of Periodontology, hyperplasia was accepted as a more accurate term (78).

Opinions varied as to the characteristic appearance of hyperplastic gingivae. Reader (77); Glickman and Lawitus (35); and Livingston and Livingston (59) were in relative agreement that the earliest manifestation of hyperplasia was a thickening of the marginal gingival tissues, mainly around the anterior teeth. According to them, the tissue became dense and pink in uncomplicated cases, and a secondary inflammation made the tissue spongy and darker pink. The hyperplasia sometimes affected all free, marginal, gingival tissue and caused interproximal tissue to swell and fill interdental spaces and eventually to cover the tooth crowns. These investigators stated that the interproximal gingival papillae became mulberry-like in texture and coalesced with labial, buccal, and lingual mucosa. Ziskin, Stowe, and Zegarelli (101) deviating from the above viewpoint, described the hyperplastic tissue in its early stage as being friable, congested, and bleeding easily if probed. This group of workers referred to a definite line of demarcation that separated the normal tissue from the adjacent hyperplastic tissue, which was first inflamed and markedly swollen, and later became hard, firm, and pink. They reported that, if secondary inflammation persisted, it was confined to the free margin of the gingiva.

The degree of hyperplasia may vary with different individuals, as well as within the same individual, and both normal and pathologic areas may be found in the same oral cavity. It has been reported that edentulous areas do not become hyperplastic. If patients with DPH hyperplasia discontinued the drug or became edentulous, the hyperplasia disappeared in 3-6 months (58, 59).

According to some investigators, microscopic investigations showed that DPH gingival hyperplasia is characterized primarily by excessive proliferation of the lamina propria; this accounts for most of the enlargement of the gingivae. Proliferative changes are also present in the epithelium. Such changes can be seen in the basal layer projecting into the underlying stroma as long, rete ridges. The number of cells in the prickle cell layer is also increased. In different specimens, the amount of inflammatory infiltrates varied according to the degree of inflammation present at the time of biopsy. In some cases of inflammation, the exudate was present as perivascular infiltrates composed of leukocytes and plasma cells. Blood vessels increased in size and numbers (35, 59, 77, 100, 101).

Earlier, it was stated that completely edentulous patients and edentulous areas are unaffected by clinical hyperplasia. However, according to the histochemical studies of Staple and Emslie (90) a mucosal reaction to DPH can occur in such instances.

Possible Etiological Factors in DPH Induced Gingival Hyperplasia

Numerous factors have been suggested as possible etiological agents, in the development of gingival hyperplasia and controversy abounds in respect to the cause of this side effect of DPH therapy.

Kimball (47) and Frankel (30) reported a relation between DPH gingival hyperplasia and the assimilation of vitamin C. Reader (77); Drake, Gruber, and Havy (22); and Ziskin, Stowe, and Zegarelli (101) were some of numberous investigators to reject the possibility of such a relation.

In historical reviews, Reader (77); Gardner, Gross, and Wynne (34); Livingston and Livingston (59); and Klar (49) cited articles related to the pros and cons of numerous factors implicated as possible etiological agents in hyperplasia. These factors include: (1) alteration in activity of the pituitary adrenal complex, (2) allergic response to DPH or to one of its metabolites, (3) oral irritations and poor oral hygiene, (4) hyperplasia induced locally by DPH or its by-products, and (5) hyperplasia induced by a low level of magnesium in the serum.

None of the several possible etiological factors tested as a provocative of DPH gingival hyperplasia has been proved to have a role in the development of this disease.

Adverse Systemic Effects of DPH Therapy

In the field of dentistry, gingival hyperplasia has received more attention than any other adverse effect resulting from DPH therapy. A recent review (5) described Dilantin as a wonder drug that is useful for treating multiple disorders. Few of the 750 references that were cited commented on any adverse effects. Livingston (57) strongly objected to articles in Life and Reader's Digest proclaiming the miraculous uses of this drug and referring to it as one of the safest drugs available. The author alluded to his personal knowledge of 20 deaths attributable to use of this drug. The product information section on Dilantin (68, 69) supplied by the manufacturer (Parke, Davis & Co.) listed numerous adverse reactions and stated that, in some instances the drug could be fatal. Lovelace and Horwitz (60) reported that 25 patients who had received DPH therapy for more than 5 years developed peripheral neuritis.

Information about still another potentially serious side effect of DPH, which has been described in recent literature, involves the administration of anticonvulsant drugs to pregnant women and the possibly related congenital abnormalities in their offspring.

Observing that epileptic mothers gave birth to an unusually large number of babies with congenital malformations (especially cleft lip and/or cleft palate) Elshove (26) investigated the effect of DPH on pregnant

mice and their offspring. He found that various DPH dosages given on different days of pregnancy caused the death of some or all of the fetuses, or produced anomalies in some of the offspring.

Harbison and Becker (39) described various teratological effects caused by different dosages of DPH administered to female mice on different days of pregnancy. Oral and subcutaneous doses produced less severe effects than those given intraperitoneally, and with some dosages death of the fetuses occurred.

Subsequent to these animal studies, reports were published describing anomalies in human infants whose mothers had received DPH therapy during pregnancy. Such reports concerned single cases or uncontrolled studies (26, 54). In a retrospective analysis of births, Dunstone (23) observed that epileptic mothers treated with DPH, prior to and during pregnancy, bore children who exhibited no higher percentage of abnormalities than children born to mothers who had not received DPH.

Effects of DPH on Connective Tissue

The failure to find suitable animal models for exploring the effects of DPH gingival hyperplasia prompted investigators (48, 91, 92, 94) to examine other factors affected by the drug, such as tensile strength of healing-wounds in animals. Wounded rats that had received DPH therapy displayed a marked increase in tensile strength, which Shafer, Beatty, and Davis (83).

attributed to an increased collagen production. Shapiro (84) showed that DPH increased the rate of gingival wound healing in nonepileptic patients and noted a microscopically detectable "increased fibroblastic activity, clot organization, and epithelial proliferation."

Houck, Jacob, and Maengwyn-Davis (42), who studied the effect of DPH on the chemistry of rat skin, reported a marked decrease in tissue water and fat; an increase in collagen, particularly in the insoluble fraction; an increase in hexosamine content; and a striking increase in the amount of noncollagenous protein. Subsequently Houck (40) reported that the level of dermal collagen was varied by the number of DPH doses administered. In instances of increased collagen levels a return to normal levels was influenced by the length of time for which the drug had been administered.

Simpson, Kuntz, and Slafta (86) described an improved condition of chronic stasis leg-ulcers after daily administration of 100-600 mg doses of DPH for 13 weeks. No statistical differences were noted in sizes of ulcers in DPH and placebo groups after treatment. Patients appeared to respond better to small than to large doses of the drug.

Sklaus, Taylor, and Shklar (87) investigated the possible beneficial effects of DPH on healing of experimentally produced fractures in rabbit mandibles.

Clot organization, connective tissue proliferation, and bone formation occurred sooner in DPH-treated animals than in controls.

Kolbert (50) reported that treatment with DPH increased tensile strength in corneal wounds of rabbits within the first week after infliction of a wound. Large doses of corticosteroids locally administered at the time of wounding prevented an increase in tensile strength. In contrast to the reported increase in tensile strength of dermal wounds in rats (83), Cheng and Staple (16) reported no differences between the rats that had received DPH prior to wounding and the controls. Houck et al. (43) stated that, with rats, the increased collagen production customarily induced by administration of DPH was prevented by some, and unaffected by other, anti-inflammatory drugs. It was noted that, if the animals were pretreated with either DPH or cycloheximide, the drug-induced loss of collagen could be completely inhibited.

In experiments with strain-L fibroblasts, Houck et al. (43) demonstrated that both proteolytic and collagenolytic activity could be induced with anti-inflammatory steroids of the 11-β-OH group, but not with any other steroids tested.

In studying the effect of DPH on collagen catabolism, Gabler (32) detected no difference in the

rate of uterine involution in rats who received DPH prior to or after delivery. Although the 2 groups had the same hydroxyproline levels, the concentration of noncollagenous nitrogen was much lower in animals treated with DPH than in control animals.

Dam and Langgard (21) found that skin collagen of swine was not significantly affected when the animals were treated with DPH for 3 or 6 months.

Ebadi and Scott (24) reported an enhanced synthesis of collagen in carrageenin-induced granulomas in guinea pigs treated with DPH. These workers suggested that increased protocollagen prolinehydroxylase (PPH) activity might be involved in the increased collagen synthesis. Liu and Bhatnagar (56) reported that, at all levels tested in vitro, DPH inhibited PPH activity. They also found that adding more Fe^{2+} could reverse the inhibition in vitro.

Effects of DPH on Cell Cultures

Shafer (80, 81, 82) reported that DPH stimulated cell growth in fibroblast-like cells, whereas it exerted no stimulatory effect on growth of cells from ectodermal organs.

Houck, Cheng, and Waters (41) and Kasai and Yoshizumi (45) noted that small dosages of DPH were more successful in stimulating cell growth and that large doses could cause cell death. Noess (67)

concurred with the findings of these authors except that he favored the use of cell suspension rather than monolayer cell cultures for kinetic studies.

Collagen Structure

Collagen, which has been described as the most abundant protein in the animal body, is the major fibrous constituent of skin, tendon, ligament, cartilage, bone, and other forms of connective tissue. Recently, much information has been published on the nature of the collagen molecule in higher animals.

Based on information obtained, between 1950 and 1970, by chemical analysis, X-ray diffraction, electron microscopy, and other techniques, the primary molecule of collagen has a molecular weight (MW) of approximately 300,000, is 2800 Å long, and 15 Å wide. This information suggested that the molecule was composed of 3 polypeptide chains of equal size, called α chains. Each of these α chains is twisted into a left-handed helix and the 3 chains of the molecule are, in turn, twisted around each other to form a right-handed super-helix. Two chains of the molecule are identical and are termed α1 chains; the third chain is different from the others, and is termed α2 (12, 33, 36, 38, 71). Collagen of codfish skin, unlike collagen from other sources, is composed of three distinct chains, termed α1, α2, and α3 (36, 51, 70, 71). At least 3 other distinct

molecular types of collagen have now been verified. The collagen of connective tissues such as that found in mature skin, bone, and tendon contains the two distinct α-chains, α1 and α2. The chain structure of this collagen, now designated $[\alpha 1(I)]_2\alpha 2$, is referred to as Type I collagen. This designation means that the molecules in these tissues are composed of two α1(I) chains and one α2 chain (65). Cartilage collagen, which is composed of 3 identical α chains, are designated as α1(II). Recent experiments have indicated that various tissues contain collagen molecules with the chain structure $[\alpha 1(III)]_3$ (18). Kefalides (46) showed that the collagen found in basement membranes is composed of 3 identical α chains that are different from those previously described and are designated as $[\alpha 1(IV)]_3$ (65). Their similarity of size and composition suggests that all α chains are homologous polypeptides. Recent amino acid sequence studies support this suggestion (13, 29, 74).

As already stated, the collagen molecule in various tissues has a MW of about 285,000 with each polypeptide chain being composed of slightly over 1,000 amino acids that have MW of approximately 95,000. Speakman (89) postulated that the newly synthesized α chain at the polyribosomal aggregate level is longer than that in the fibrillar form, and that such an extended peptide would probably be on the N-terminal end and thus could

aid in forming a triple helix by properly registering the polypeptides. Speakman suggested that this extended (registration) peptide might be removed later by enzymatic action and that a defective excision enzyme(s) might cause a connective-tissue disorder.

Only recently, the suspected synthesis of a precursor form of collagen was verified by examining the medium of fibroblasts in culture (52). Called procollagen (4), this form of the molecule has not been clearly elucidated as to its exact nature and function, but it appears to be comprised of chains approximately 15-20% larger than α chains. The amino acid composition of the extended portion of the procollagen molecule is quite different from the remainder of the chain (10, 25, 78, 88, 98). Recent studies have shown that certain types of abnormal collagen found in cattle (53, 98), sheep (65), and humans (55) are composed of extended chains.

Evidence has been presented to show that the individual procollagen chains form disulfide-linked trimers (pro γ112) (28) and are then secreted into the extracellular matrix. This extended portion of the secreted form is apparently cleaved through a series of steps during which collagen is formed.

Cyanogen Bromide (CNBr) Digests of Collagen

Early attempts at characterizing the primary structure of collagen were plaqued with difficulty

because of its insolubility and its molecular composition. In early studies, partial acid hydrolytic and enzymatic methods were used to characterize whole collagen. The only information obtained by these methods concerned random short regions of the molecule. With this approach, it would have been an almost insurmountable task to characterize the α chains in collagen (6, 11, 97). Methods for isolating more individual α chains greatly simplified the identification (8, 72).

After purified α chains were obtained, CNBr, which cleaves peptides at methionyl residues (37), yielded specific degradations of purified α chains to polypeptides of more workable sizes. These reactions were carried out in 0.1 N HCl at 30°C for 4 hours using equal weights of α chains and CNBr (9). The kinetics and mechanism for this reaction are specific and sensitive enough to be a valuable tool in such protein investigations (44).

The major vertebrate collagens contain 5-9 residues of methionine per α chain; therefore, the reaction with CNBr should yield 6-10 peptides per chain. The CNBr peptides from several animal species have been isolated and characterized by MW and amino acid compositions.

> The nomenclature of the peptides is based on the results obtained for rat skin (or tendon) collagen. They were designated according to the chain of origin and were assigned numbers in the order in which they appeared in the effluent of the ion-exchange column. Peptides from other collagens were assigned numbers on the basis of homology to the peptides from rat

> collagen. If a methionine was absent, giving rise to one peptide equivalent to two peptides from rat collagen, both numbers separated by a comma were used. For example, α1-CB (0,1) from human skin collagen is homologous to α1-CB0 and α1-CB1 from rat tendon collagen. If an extra methionine was present, the letters A and B were used. For example, α1-CB6A and α1-CB6B from chick skin and bone collagen are homologous to α1-CB6 from rat skin collagen.....(97).

The CNBr peptides can now be identified according to their positions of origin in the parent α chains by the use of chemical, electron microscopic, and pulse labeling data (75, 97).

After adequate characterization of peptides from isolated, individual, soluble chains, it was possible to undertake the more complex task of characterizing insoluble collagens (27).

CNBr peptides from cartilage collagens of chicks (62) and of bovine and humans (64) have been characterized. These peptides were homologous and contained only minor differences in amino acid composition (64).

The CNBr peptides from soluble and insoluble human skin collagen have been isolated and characterized (20, 63). Significant levels of peptides identified as α1(III) were detected in studies on insoluble collagen of infant dermis (63). More recent studies have shown that many human tissues contain molecules with the chain structure $[\alpha 1(III)]_3$ (17, 18).

Acidic, Noncollagenous Proteins of Connective Tissues

After treating rats with DPH, Houck, Jacob, and Maengwyn-Davis (42) reported finding a unique noncollagenous protein in rat skin. These investigators stated that the size and number of DPH doses determined the amount of and the period for which this noncollagenous protein (referred to as scleroprotein) was present (40).

Timpl, Wolff, and Weiser (95) reported their isolation of a possibly new class of structural protein, which contained no hydroxyproline, little glycine, and a high concentration of glutamic and aspartic acid groups (96). It was further stated that this heterogeneous material was joined partially by disulfide bonds (31). It was concluded that some of the heterogeneity might have stemmed from polymerization of a subunit polypeptide chain, or that the partial degradation might have resulted from the harsh treatments required to solubilize the material (99).

Gingival Collagen

Little biochemical information is available on the collagenous fibers found in gingival and other periodontal structures.

Schultz-Haudt and Aas (79) reported no appreciable differences in amounts of soluble collagen present in

clinically normal and chronically inflamed tissues. On the other hand, Stern (93) obtained an increased level of salt-soluble collagen from inflamed edematous gingivae. An increased percentage of acid-soluble collagen was obtained from inflamed fibrotic gingivae (including tissue from patients with DPH hyperplasia).

MATERIALS AND METHODS

Source and Preparation of Tissues

Dilantin hyperplastic gingivae (DHG) were obtained from 10-20 year old DPH-treated patients (3) (Figures 1 and 2). This group of patients (mixed as to age, sex, and race) was receiving DPH treatment for seizure disorders. Their clinical degree of hyperplasia ranged from moderate (Figure 1) to severe (Figure 2). Most samples were obtained from severely affected patients. These specimens represented various microscopic patterns with differing degrees of inflammation present.

At postmortem examination of term, stillborn infants (SI), normal alveolar gingivae were obtained from that portion of oral mucosa overlying the alveolar ridges. Attached bovine gingivae were obtained at a local slaughter house from animals aged 6-24 mo. Palatal mucosa was excluded from SI and bovine samples. Gingival specimens were frozen and stored for future use.

Before the extractions were begun, the tissues were thawed, washed briefly with several changes of distilled water, lyophilized, and weighed. The tissue was cut into small pieces by a procedure similar to that used used by Epstein et al. (27), instead of being ground into a powder (7).

After rehydration the tissues were carried through the following procedures: extracted with 10% NaCl in 0.05 M Tris-HCl (pH 7.4); washed with 3 changes of cold distilled water; extracted with 0.5 M acetic acid; washed thoroughly with distilled water; delipidated by sequential washings with acetone, ether, acetone, and water; extracted with 0.15 M sodium phosphate (pH 7.4); washed with 3 changes of water; reextracted with 0.5 M acetic acid; and again washed with distilled water. Re-extractions were performed with the 10% NaCl in 0.05 M Tris-HCl (pH 7.4). The specimens were then thoroughly washed with distilled water, lyophilized, and weighed again. The volume of extractants in ml was 10 times that of the original wet weight of the tissue in g. All the extractions were for 2 day periods at 4°C except for the delipidation steps, which were for 4 hours each.

Each extract was lyophilized, placed in a small volume of distilled water (20-40 ml), and transferred to dialysis tubing (Sargent-Welch, C-65). After exhaustive dialysis against distilled water, each extract was lyophilized, weighed, and then analyzed for hydroxyproline by use of an amino acid analyzer.

The residue that remained after numerous extractions was lyophilized and weighed. This material, referred to as insoluble gingival collagen (IGC), was used as starting material for the subsequent experiments.

Cyanogen Bromide (CNBr) Treatment of Insoluble Gingival Collagen

Samples of IGC were suspended in 70% formic acid at a concentration of 10 mg/ml, flushed with nitrogen, then placed on a magnetic stirrer and incubated with a hundred-fold excess (relative to methionine) of CNBr at room temperature for 4 hr.

The unreacted IGC material (usually less than 5%) was removed by centrifugation, and the solubilized CNBr peptides were desalted by gel filtration on columns of Bio-Gel P2 to remove the acid and unreacted CNBr. After lyophilization and weighing, samples of the CNBr peptides were hydrolyzed for amino acid analyses before separation of the various peptides.

CM-Cellulose Chromatography

Samples of CNBr digest (100-200 mg) were initially chromatographed on a jacketed column (2.5 x 15 cm) of CM-cellulose (Whatman 32 microgranular, capacity 1.0 meq/g) at 40°C. Samples were dissolved in 15 ml of 0.02 M sodium citrate, pH 3.6 (16, 30). A significant amount of this material was insoluble in the 0.02 M sodium citrate and was removed by centrifugation (10,000 g for 30 min) and saved for future experiments. The soluble portion of the sample was pumped onto the column with a Buchler polystaltic pump and immediately afterward the column was eluted with 50 ml of 0.02 M sodium citrate

(pH 3.6) and then eluted with a linear gradient at a flow rate of 200 ml per hr. The linear gradient was formed from 1100 ml each of 0.02 M sodium citrate (pH 3.6) containing 0.02 M NaCl as starting buffer and 1100 ml 0.02 M sodium citrate (pH 3.6) containing 0.14 M NaCl as limit buffer. The effluent, constantly monitored with a Beckman DB-GT spectrophotometer equipped with a flow cell, was collected in 15 ml fractions on an LKB Ultrorac 7000 fraction collector.

The CM-cellulose columns were replaced by a 2.6 x 16 cm jacketed column from Pharmacia in later runs that were carried out under the conditions just described.

Further separations of peptides in some zones were performed on the CM-cellulose columns by use of a concave gradient system (14). In such cases the columns were equilibrated with starting buffer (0.02 M sodium acetate, pH 4.8) and eluted with a concave gradient formed by 1000 ml starting buffer and 740 ml 0.02 M sodium acetate (pH 4.8) containing 0.14 M NaCl as the limit buffer. The conditions for these runs were similar to those used in the linear gradient runs described.

Molecular Sieve Chromatography

Pooled fractions, or zones (Figures 8, 9, 10) were lyophilized, dissolved in 20-30 ml 0.2 M acetic acid and desalted on a Sephadex G-25 (coarse, exclusion

10,000) column equilibrated with 0.2 M acetic acid. The zones that contained the smaller peptides were desalted either on a Sephadex G-50 (fine) or with a Bio-Gel P-2 (100-200 mesh, exclusion limit 1800) column eluted with 0.2 M acetic acid. The peptides were lyophilized and weighed.

A gravity flow, 1.8 x 135 cm column of Bio-Gel A-1.5 M (200-400 mesh, range 10,000-1,500,000 mw) was used to estimate MW and, in some cases, to further purify the peptides. The column was equilibrated and eluted with 1.0 M $CaCl_2$-0.05 M Tris-HCl (pH 7.4) at room temperature. This procedure (73) was modified by using glycine instead of ^3H-water. From each fraction, 0.2 ml samples were taken to determine ninhydrin-positive material. A Technicon Auto-analyzer was used.

Phosphocellulose Chromatography

Several peptides were separated by chromatography on a column of phosphocellulose (Whatman, floc, bulk, 140-200 mesh) operated at 40°C (15). Different types of columns were tried with varying degrees of success. The phosphocellulose packed progressively tighter which necessitated repacking the column before each run. Better runs were obtained with a jacketed column (Pharmacia, 1.5 x 10 cm) packed with phosphocellulose Bio-Rad Cellex-P, exchange capacity 0.87 meq/9, 140-200 mesh). This column allowed for back-washing

which helped to prevent excessive packing.

In the first of 3 buffer systems tried, the column was equilibrated with 0.001 M sodium formate (pH 3.6) containing 0.1 M NaCl as starting buffer and eluted with a linear gradient of 750 ml each of the starting buffer and 0.001 M sodium formate (pH 3.6) containing 0.8 M NaCl as the limit buffer (15).

In a second system, the column was equilibrated with a starting buffer of 0.001 M sodium citrate that contained 0.1 M NaCl and eluted with a linear gradient of 500 ml each of starting buffer and 0.001 M sodium citrate (pH 3.6) that contained 0.8 M NaCl as limit buffer.

In the third system the column was equilibrated with a starting buffer of 0.001 M sodium acetate (pH 4.8) (27) and eluted with 500 ml each of starting buffer and 0.001 M sodium acetate (pH 4.8) with different concentrations (0.0 to 0.8 M) of NaCl as limit buffer.

Amino Acid Analysis

Samples were hydrolyzed in constant-boiling HCl at 108°C for 24 hr in a nitrogen atmosphere. Analyses were performed on a Beckman 120C automatic amino acid analyzer, modified for single-column, high speed analysis (66). Corrections were made for losses of threonine, serine, tyrosine, and valine (76).

Solubility Studies

The extractants from the preparation of the IGC were lyophilized, dialyzed, and weighed. Samples of each extractant were hydrolyzed and placed on an amino acid analyzer. The composition was calculated in residues per 1000. The number of hydroxyproline residues per 1000 amino acids, divided by the number found in pure collagen was used to calculate the amount of collagen solubilized during each extraction step. For example, if 0.020 g of material (dry weight) that was extracted with 0.5 M acetic acid contained 50 hydroxyproline residues/1000 amino acids (assuming pure collagen contains 100/1000) approximately 50%, or 0.010 g, of the extracted material would be collagen.

RESULTS

Clinical and Microscopic Features of DPH Gingival Hyperplasia

Figure 1 illustrates moderate gingival hyperplasia secondary to DPH therapy. The clinical photograph portrays generalized hyperplasia of the free and attached gingivae similar to that previously described (39, 64, 84). The interproximal tissue is bulbous but nearly normal in color. The crowns of the teeth are partially covered with the hyperplastic tissue. In such case, the overgrowth of tissue makes proper oral hygiene difficult. Generalized materia alba is present at the interproximal areas of lower anterior teeth and along cervical margins of other teeth.

Figure 2 depicts a 19-yr-old female who experienced severe gingival hyperplasia after long-term DPH therapy. This clinical photograph and an intraoral view of the same patient (Figure 3), illustrate an extreme example of DPH gingival hyperplasia, wherein the disease has progressed so that almost the entire crowns of the teeth are covered. The patient was unable to close her mouth and mastication was impossible. In this case, the hyperplastic tissue was firm and nonfriable; in areas of previous trauma, the tissue was hemorrhagic. The maxillary

right buccal area shows the mulberry-like appearance that has been described (84). Figure 4, which shows the microscopic appearance of a biopsy specimen from the patient in Figure 2, reveals marked fibrosis of the connective tissue. Figure 5 shows increased vascularity of the specimen. These sections also depict epithelial hyperplasia with marked elongation of the rete ridges. No inflammatory infiltrates are evident in any photomicrographs of the sections shown (Figures 4-7). In other sections examined (not illustrated) minimal inflammatory infiltrates were evident, but the infiltrate was restricted to perivascular areas. Figure 7 illustrates an unexplained area of epithelial keratinization in one of the elongated rete ridges. This type of area has been described as an epithelial pearl. Fibroblasts in this photomicrograph appear to be normal, mature cells.

Solubility of Gingival Collagen

Table 2 shows the extent of the solubility of collagen from samples of DPH and SI gingivae along with relative dry weights of the fractions tested. Tables 3 and 4 give the results of amino acid analyses for each of these fractions. Four per cent (Table 2) of the gingival collagen from patients on DPH therapy was soluble as compared with 17% from SI. The first 0.5 M acetic acid extraction yielded a larger amount of soluble collagen from SI than did any other extractant. In both types of tissue, most of the collagen was insoluble in

the extractants, but the procedure resulted in a significant degree of purification, as evidenced by the changes in amino acid composition.

The glycine and hydroxyproline contents of the insoluble preparations from DPH and SI gingivae strongly imply that these preparations were comprised principally of collagen (Table 5). The composition of insoluble collagen from gingivae of SI was similar to that from infant skin (7, 27). In the DPH preparations, glycine, hydroxyproline, and proline contents were lower, whereas cysteine, tyrosine, and histidine levels were higher than those in the SI preparations. This finding suggested that the preparation from the DPH gingivae was richer in noncollagenous proteins than was that from SI gingivae.

CNBr Peptides

After initial fractionation, the profiles of CNBr peptides from collagen were obtained by CM-cellulose chromatography (Figures 8, 9, 10). Profiles of these peptides resembled those from preparations of skin collagen. One striking difference between the chromatograms of the skin and the gingivae was the appearance on the latter of additional peptide peaks that eluted between 700 and 900 ml. When the same chromatographic conditions were used, similar components have since been observed in experiments with insoluble rat skin collagen (W. T. Butler and J. E. Finch, Jr., unpublished data). These peptides do not stem from either $\alpha 1(I)$ or

α2 chains.

Rechromatography of fractions from each peptide peak (CM-cellulose runs) led to separation of CNBr peptides that represented essentially all of the α1(I) and α2 chains. However, by the methods thus far attempted, it was not possible to isolate the small peptides α1(I)-CB(0,1), α2-CB0, and α2-CB1 in a pure enough form for unequivocal identification. The difficulty arose partly from the limited amount of starting material. Amino acid analyses of the purified peptides from α1(I) and α2 (given in Tables 7 and 8, respectively) accounted for about 95% of the α1(I) and 99% of the α2 chains. These analyses indicated that all peptides, except for α1-CB3, were in relatively pure form. Any lack of purity was reflected in the difficulty of obtaining whole integers when calculating the number of residues per peptide.

One small peptide, α2-CB2, was purified by gel filtration on Sephadex G-50 (14); a second peptide, α1(I)-CB2, was purified by ion-exchange chromatography on phosphocellulose (15). The fractions containing peptides α1(I)-CB3, α1(I)-CB4, and α1(I)-CB5 were initially separated on Sephadex G-50 (Figure 11) and rechromatographed on phosphocellulose (Figure 12) before amino acid analysis. Subsequently, better separations were obtained by ion-exchange chromatography on CM-

cellulose with sodium acetate (pH 4.8) buffers utilizing a concave gradient system. Further purification was then carried out on a phosphocellulose column that was eluted by either an acetate or formate buffer system. The larger peptides, α1(I)-CB6, α1(I)-CB7, and α1(I)-CB8 were rechromatographed on CM-cellulose with sodium acetate (pH 4.8) buffers by use of a concave gradient (15). Three larger peptides from α2 were rechromatographed successively on CM-cellulose in sodium acetate (pH 4.8), phosphocellulose, and Bio-Gel A-1.5 M (73).

Only 2 CNBr peptides from α1(III) have been isolated (chromatographed by concave CM-cellulose and then by phospho-cellulose techniques) in pure enough form to be identifiable in fractions eluting at 490-550 ml and at 710-860 ml (Figure 8). The composition of peptides from these fractions was similar to that of peptides derived from human α1(III) chains (17, 18). The amino acid analyses for 2 representative α1(III) peptides from human gingivae are given in Table 9.

Noncollagenous Acidic Proteins

Prior to CM-cellulose chromatography of the peptides solubilized by the reaction of CNBr with the insoluble collagen preparations, the material was normally dissolved in 15 ml of 0.02 M sodium citrate (pH 3.6). In this study, it was noted that a substantial proportion of the material from gingivae would not redissolve in

the citrate buffer. These undissolved fractions consistently accounted for 20% of the dry weight of the DPH collagen preparation as opposed to 6% of that of SI collagen. Amino acid analysis (Table 6) indicated the presence, in these fractions, of small amounts of CNBr peptides derived from collagen and larger amounts from noncollagenous proteins. The noncollagenous protein fractions from DPH and SI tissues were almost identical in composition, but both had lower levels of glycine, proline, and hydroxyproline and higher levels of aspartic and glutamic acid residues than did collagen. A significant amount of cysteine was also present in DPH and SI noncollageneous proteins. This amino acid is not normally present in Type I collagen (20).

Preliminary studies of insoluble collagen from human gingivectomy specimens (gingivae excised in the course of treatment of chronic periodontal disease) revealed that 34% of this CNBr digest and 32% of the CNBr digest from bovine insoluble gingival preparation were insoluble in the 0.02 M sodium citrate (pH 3.6) buffer. The significance of these levels of noncollagenous proteins would have to be correlated with the results of solubility studies before any conclusions could be drawn.

Although the characterization of the noncollagenous proteins (material insoluble in 0.02 M sodium citrate,

pH 3.6) was beyond the scope of this investigation, certain preliminary steps showed that these proteins were also insoluble in all the other starting buffer systems (see Materials and Methods). However, the noncollagenous proteins were soluble when placed in H_2O and titrated with NaOH to a pH above 8 or 9. When the pH was lowered with 0.1 N HCl, the proteins precipitated from solution below a 6-7 pH range.

The proteins were solubilized in buffers above pH 9.0 and chromatographed on a DEAE ion-exchange column. Four UV-absorbing peaks were obtained: a large one in the void volume of the column and 3 minor peaks that were eluted later. The large peak was desalted, lyophilized, and found to be soluble in 1 M $CaCl_2$-0.05 M Tris-HCl, pH 7.4. This noncollagenous material, when subjected to gel filtration on Agarose Bio-Gel A-1.5 M, yielded 10 peaks with MW ranging from 1,700-120,000.

In preparing this material for histological examination, it was found to be immediately soluble in the 10% neutral formalin solution normally used for fixing specimens in histology. The material was then fixed in 70% ethanol, embedded, sectioned, and stained with H. & E. Only nondescript amorphous material was found in the section.

Samples of the noncollagenous protein material were examined by electron microscopy for possible accumulation of oxytalan fibers (85). These sections revealed a few cross-striated fragments that resembled short segments of collagen fibers, but most of the material was amorphous in character.

DISCUSSION

This study was undertaken with the aim of characterizing gingival, collagen, which was shown to be primarily of the $[\alpha 1(I)]_2\alpha 2$ type. Evidence is presented that molecules with the chain composition $[\alpha 1(III)]_3$ are also present in this tissue. No significant differences were noted between the CNBr peptides of the SI, DPH, or human-skin collagens; therefore, the collagen that accumulates in the hyperplastic gingivae after DPH therapy, is probably of normal quality.

In the present study, the 17% solubility of the collagen in gingivae from SI (Table 2) was similar to the solubility reported for normal gingivae. However, Stern's report of 40-70% solubility of the collagen from DPH gingivae contrasts sharply with the 4% noted in the current investigation (93). The use of different techniques and the differences in the condition of the tissues examined in the two studies, necessarily limited comparison.

Of the various types of gingivae tested, that from patients who had periodontal disease or had received DPH therapy, exhibited the highest levels of non-collagenous proteins. The compositions of the

noncollagenous proteins in DPH and SI gingivae were found to be similar (Table 6). But, since the variables, age and inflammatory state, were not controlled, data presented here do not allow an unequivocal conclusion that DPH therapy leads to a pathologic increase of noncollagenous proteins in gingivae. It should be noted that these proteins appeared similar in composition to the acidic structural proteins found in skin (95, 96, 99). No function has as yet been assigned to these substances.

The hyperplastic morphology of specimens made it impossible to remove the epithelial layer during the preparation of the insoluble collagen. Since the hyperplasia also affects the epithelium, examination of this layer would be necessary to determine whether it might be a possible source of the noncollagenous material. Thus far, the increase in noncollagenous material and the lack of hyperplasia in the epithelial layer of gingivae from patients who have undergone gingivectomy (for periodontal disease) seem to discourage an assumption that the epithelial layer is the source of noncollagenous proteins. It is likely that this question will be resolved by ongoing experiments, wherein the epithelial layer of bovine gingivae can be separated from the underlying connective tissue after the sequential extractions and lyophilizations have been performed.

The recent finding that the newly synthesized collagen chain (procollagen) contains extension (registration) peptides arouses conjecture as to a possible relationship between these peptides and the insoluble acidic protein(s) found in the gingivae. The insoluble noncollagenous protein, which is intimately associated with the insoluble collagen, displays some of the following compositional features that have been reported for fragments of the procollagen extension peptides: low hydroxyproline; a content of less than one third glycine; high aspartic and glutamic acid content; and the presence of half-cystine (98). Preliminary evidence against such a relationship would be the apparently heterogeneous nature of the peptides in noncollagenous proteins from gingivae. However, the heterogeneity could represent fragmentation products; therefore, further investigation would be needed to answer this question.

Several current and projected studies have evolved from this investigation. In addition to efforts to further characterize the peptides found in the noncollagenous gingival material, studies are presently being conducted on collagen of bovine gingivae to isolate and complete the characterization of the α1(III) collagen.

SUMMARY

The biological significance of collagens in the various connective tissues has been increasingly stressed by current research. Despite the wealth of available information concerning the composition of collagen and other connective tissue elements, no definitive studies have been reported on the chemical character of the fibers in gingivae and other periodontal structures.

Gingivae were obtained from 10-20 year old, DPH-treated patients. Normal alveolar gingivae were obtained from term, stillborn infants (SI). Insoluble, whole-collagen preparations were obtained by sequential neutral-salt and dilute-acid extractions. Experiments were performed to determine the solubility of collagen from both types of tissue. In this study, 4% of the gingival collagen from patients on DPH therapy was soluble, as opposed to over 17% from SI. The collagen from the latter was more soluble in 0.5 M acetic acid than in other extractants.

The insoluble collagen was solubilized by reacting it with CNBr. The resultant CNBr peptides, isolated and purified by various ion-exchange and molecular-sieve chromatographic procedures, were further characterized by amino acid analyses. Most of the CNBr peptides from

α1(I) and α2 chains of SI- and DPH collagen were isolated and characterized. The composition of these peptides was identical, or nearly identical, to that reported for peptides derived from human-skin collagen. In the chromatographic patterns and in the amino acid analyses of the peptides, no differences were noted between the insoluble collagens from SI and from DPH hyperplastic gingivae. Some of the CNBr peptides that were obtained had a similar composition to that of peptides derived from human α1(III) chains. Experiments reported here demonstrate gingival collagen to be primarily of the $[\alpha 1(I)]_2\alpha 2$ type and suggest that molecules with the chain structure $[\alpha 1(III)]_3$ are present in this tissue. Collagen that accumulates as a result of DPH hyperplasia is probably of normal quality.

The composition of noncollagenous proteins is similar in DPH and SI gingivae, but the levels are strikingly higher in the former. Because such variables as age and inflammatory state were not controlled in this study, the data do not allow an unequivocal conclusion that DPH therapy leads to a pathologic increase of noncollagenous proteins in gingivae.

APPENDICES

Table 1.

Incidence of gingival hyperplasia in patients who received diphenylhydantoin (DPH) therapy. Data were obtained from recent reviews (2, 84).

Year	Investigator	Number of Patients	% Showing Gingival Changes
1939	Hodgson and Reese	44	7
1939	Blair, Bailey and McGregor	75	2.7
1939	Hodgson and Reese	88	3.4
1939	Kimball	119	57.1
1939	McCartan and Carson	20	5.0
1939	Merritt and Putnam	350	6.0
1939	Pratt	52	50.0
1939	Steel and Smith	20	15.0
1939	Williams	91	0
1940	Weinstein and Goldstein	15	33
1940	Bergman	57	14.0
1940	Butter	43	2.3
1940	Fetterman	28	25.0
1940	Frankel	48	62.5
1940	Merritt and Foster	182	22.0
1940	Schlotthauer	32	12.2
1941	Blickman and Lewitus	76	21.0
1941	Lowry	34	29.0
1941	Ziskin, Stove and Zegarelli	18	55.0
1942	Millhon and Osterberg	30	40
1942	Robinson	143	19
1942	Macfarlane, Baxter and Mitchell	67	55
1942	Prudhomme	57	30
1942	Polk	20	50
1942	Finkelman and Ariefi	44	38.6
1943	Boller	67	0
1943	McLendon	29	3.4
1943	Stern, Eisenbud, and Klatell	50	52.0
1943	Bindslev and Stubbe Tegibjaerg	130	42.3
1945	Esterberg and White	244	54.0
1946	Bergman	86	54.6
1949	Scarella and Belletti	38	76.3
1954	Dummett	123	38.2
1955	Spira	52	69.2
1960	Collins and Fry	50	64.0
1960	Panuska et al.	546	32.0
1962	Gardner, Gross and Wynne	77	78.0

Table 1 - continued

Year	Investigator	Number of Patients	% Showing Gingival Changes
1965	Babcock	369	35.5
1965	Winthrop and Kapur	137	72.0
1966	Wolf	100	36.0
1968	Tollaro	71	84.5
1968	Al-Safi	129	58.0
1969	Love	61	65.0
1972	Angelopoulos and Goaz	173	53.2

Table 2.

Solubility studies of DPH and stillborn infant (SI) gingival collagen.

Sequential Extractant[b]	TISSUE			
	DPH		SI	
	Dry Weight (%)	Collagen[a] (%)	Dry Weight (%)	Collagen[a] (%)
10% NaCl, .05 M Tris-HCl, pH 7.4	13.9	0	14.7	1.6
0.5 M Acetic Acid	1.1	0.82	11.2	11.3
0.15 M Sodium Phosphate, pH 7.4	1.5	2.5	3.2	3.3
0.5 M Acetic Acid	0.5	0.54	0.77	0.77
10% NaCl, 0.05 M Tris-HCl, pH 7.4	0.4	0.18	0.60	0.47
Total	17.6	4.04	30.5	17.44
Insoluble Collagen	51	96	62	82.6

a. Calculated from the relative hydroxyproline content of each fraction.

b. See Materials and Methods for details.

Table 3.

Amino acid composition of DPH gingival extracts.

Amino Acids	Sequential Extractant*				
	10% NaCl 0.05 M Tris-HCl pH 7.4	0.5 M Acetic Acid	0.15 M Sodium Phosphate pH 7.4	0.5 M Acetic Acid	10% NaCl 0.05 M Tris-HCl pH 7.4
	Residues given per thousand				
3-Hydroxyproline	-	-	Trace	-	-
4-Hydroxyproline	-	28.7	62.5	39.3	20.4
Aspartic Acid	101	82.4	60.5	79.4	94.3
Threonine	61	39.9	31.4	39.7	53.3
Serine	71.5	68.8	48.2	68.2	68.6
Glutamic Acid	115.5	117.5	85.7	113.6	105.6
Proline	35.8	62.6	108.5	52.6	58.5
Glycine	85	187.1	264.2	188.7	168.7
Alanine	104.5	95.2	111.4	89.0	131.8
Half Cystine	10.4	9.3	4.3	12.2	5.0
Valine	82.2	43.4	35.8	50.1	39.0
Methionine	19	16.1	19.0	15.7	19.2
Isoleucine	33.9	23.8	16.2	23.9	22.6
Leucine	91.4	74.7	40.5	73.2	68.2
Tyrosine	30.6	16.0	8.4	15.6	21.6
Phenylalanine	36.5	27.8	18.2	27.0	26.3
Hydroxylysine	-	3.0	4.2	31.1	3.5
Lysine	60.2	43.6	34.2	46.2	47.4
Histidine	14.1	13.7	8.7	10.9	11.0
Arginine	41.6	48.9	46.0	51.3	39.8
Amino Sugars	Present	Absent	Trace	Present	Absent

* See Materials and Methods for Details.

Table 4.

Amino acid composition of SI gingival extracts.

Amino Acids	Sequential Extractant*				
	10% NaCl 0.05 M Tris-HCl pH 7.4	0.5 M Acetic Acid	0.15 M Sodium Phosphate pH 7.4	0.5 M Acetic Acid	10% NaCl 0.05 M Tris-HCl pH 7.4
	Residues given per thousand				
3-Hydroxyproline	-	1.8	Trace	.6	Trace
4-Hydroxyproline	14.1	65.6	67.5	64.7	46.6
Aspartic Acid	90.8	52.5	64.8	65.0	74.9
Threonine	48.8	23.8	37.4	30.2	42.0
Serine	78.0	37.2	45.4	44.5	57.8
Glutamic Acid	132.0	113.7	88.2	91.5	94.9
Proline	50.0	94.1	104.9	70.5	92.2
Glycine	124.2	333.5	255.9	256.3	252.0
Alanine	82.0	106.7	106.2	111.3	122.0
Half Cystine	11.2	-	5.0	2.9	-
Valine	54.0	26.0	28.8	50.0	53.0
Methionine	20.9	1.4	27.1	12.4	11.8
Isoleucine	26.0	17.9	15.2	18.2	19.3
Leucine	89.9	33.4	39.9	45.1	47.3
Tyrosine	25.0	-	8.9	9.7	11.5
Phenylalanine	33.2	12.3	18.1	19.9	22.5
Hydroxylysine	1.0	2.6	5.4	4.2	3.5
Lysine	55.2	29.3	33.8	37.8	39.0
Histidine	16.6	7.1	8.8	7.1	9.3
Arginine	46.5	40.9	44.5	48.6	46.7
Amino Sugars	Present	Present	Present	Present	Absent

* See Materials and Methods for details.

Table 5.

Amino acid composition of insoluble collagen from DPH and SI gingivae.

Amino Acid	SI	DPH
	Residues given per thousand	
3-Hydroxyproline	0.4	0.8
4-Hydroxyproline	93	66.9
Aspartic Acid	49.1	67.6
Threonine	19.3	31.4
Serine	38.6	57.5
Glutamic Acid	78.5	99.3
Proline	115	87.0
Glycine	321	238
Alanine	105	95.6
Cysteine	0.5	4.8
Valine	23.7	32.8
Methionine	9.8	12.5
Isoleucine	11.6	18.5
Leucine	28.5	48.6
Tyrosine	5.1	12.7
Phenylalanine	14.0	21.0
Hydroxylysine	7.8	6.0
Lysine	26.5	37.9
Histidine	5.9	10.8
Arginine	47.4	50.0

Table 6.

Amino acid composition of noncollagenous proteins.

Amino Acid	SI	DPH
	Residues given per thousand	
4-Hydroxyproline	18.3	17.6
Aspartic Acid	84.7	92.5
Threonine	48.7	65.6
Serine	84.0	72.4
Homoserine	11.6	7.0
Glutamic Acid	111	118
Proline	54.6	69.6
Glycine	142	129
Alanine	72.6	75.0
Cysteine	12.3	14.0
Valine	56.7	50.8
Isoleucine	32.6	33.6
Leucine	80.8	81.1
Tyrosine	33.0	30.8
Phenylalanine	33.5	34.0
Hydroxylysine	3.1	3.4
Lysine	46.0	42.5
Histidine	12.4	13.9
Arginine	52.7	50.2

Table 7.

Amino acid composition of peptides from α1(I) chains of DPH gingival collagen.

Amino Acid	α1-CB2	α1-CB3	α1-CB4	α1-CB5	α1-CB6	α1-CB7	α1-CB8
	Residues given per peptide						
3-Hydroxyproline	-	-	-	-	0.8	-	-
4-Hydroxyproline	5.0	16.1	5.0	3.0	19.6	27	29
Aspartic Acid	-	5.9	3.0	3.1	8.8	11	10.5
Threonine	-	-	1.0	-	3.9	5.2	5.0
Serine	2.0	2.9	-	2.0	8.3	13.4	7.9
Homoserine	1.0	1.0	1.0	1.0	-	1.0	1.0
Glutamic Acid	4.1	15.9	3.0	3.2	14.0	19.0	20.0
Proline	7.0	15.0	6.0	2.2	26	39	30
Glycine	11.4	49	16.0	12.7	69	88	86
Alanine	2.0	21	3.0	4.1	19	34	33
Valine	-	4.1	-	-	3.8	5.8	6.0
Isoleucine	-	-	-	-	2.2	2.6	1.5
Leucine	1.0	3.2	2.0	1.0	3.9	4.1	4.0
Phenylalanine	1.0	2.5	-	0.9	1.2	3.3	2.8
Hydroxylysine	-	0.5	-	1.6	1.2	1.1	1.4
Lysine	-	4.5	2.0	1.4	6.4	9.4	8.6
Histidine	-	-	-	0.8	0.2	-	0.2
Arginine	1.0	6.0	4.0	1.2	8.2	14.0	15.0
Total*	35	148	46	38	196	277	262

* Summation of nearest integrals for the amino acid residues.

Table 8.

Amino acid composition of peptides from the α2 chains of DPH gingival collagen.

Amino Acid	α2-CB2	α2-CB3	α2-CB4	α2-CB5
	Residues given per peptide			
3-Hydroxyproline	-	-	-	0.5
4-Hydroxyproline	2.8	32.9	32.3	26.7
Aspartic Acid	2.1	12	13.5	15.0
Threonine	-	5.9	5.9	5.9
Serine	2.0	10.0	11.8	12.0
Homoserine	0.9	1.0	1.2	0
Glutamic Acid	1.0	24	23.0	21.5
Proline	3.0	35	37.9	36.0
Glycine	10.1	108	105	104
Alanine	3.0	39	37.0	30
Valine	1.0	8	7.5	8.8
Isoleucine	-	2	2.5	4.1
Leucine	0.9	4	8.3	8.1
Phenylalanine	-	3.4	5.0	3.7
Hydroxylysine	-	1.7	3.4	2.5
Lysine	-	9.8	7.5	5.7
Histidine	-	1.0	1.9	3.2
Arginine	2.9	16.	17.8	15.4
Total*	30	314	322	304

* Summation of nearest integrals for the amino acid residues.

Table 9.

Amino acid composition of peptides from the α1(III) chain of DPH gingival collagen.

Amino Acid	α1(III)-peptide 2[a]	α1(III)-peptide 6[a]
	Residues given per peptide	
4-Hydroxyproline	7.0	11.7
Aspartic Acid	1.0	4.0
Threonine	-	0.8
Serine	4.2	4.1
Homoserine	1.0	0.8
Glutamic Acid	2.8	7.1
Proline	5.5	5.6
Glycine	12.7	30.3
Alanine	.5	9.4
Valine	1.0	1.0
Isoleucine	-	0.8
Leucine	-	0.9
Phenylalanine	-	0.8
Hydroxylysine	-	0.6
Lysine	1.0	1.2
Histidine	-	0.8
Arginine	2.0	5.0
Total[c]	38	86

a. Nomenclature is taken from Chung et al., (1974).

b. Sum of homoserine and homoserine lactone.

c. Summation of nearest integrals for the amino acid residues.

Figure 1.

An 11-year-old female with moderate gingival hyperplasia secondary to DPH therapy.

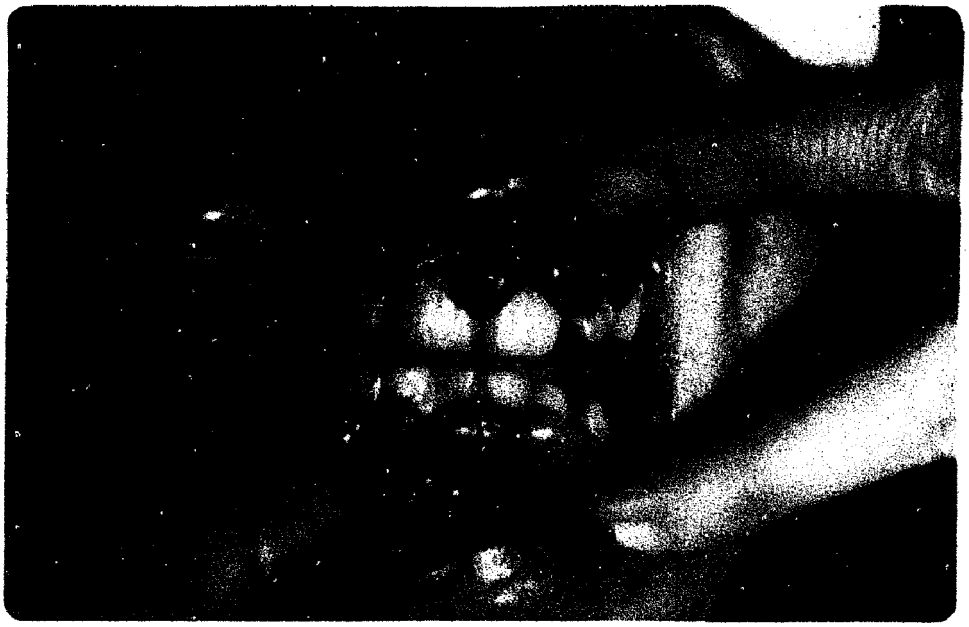


Figure 2.

Severe gingival hyperplasia in a 19-year-old female on long-term DPH therapy. Examples in Figures 1 and 2 illustrate extremes of tissue types used in this study.

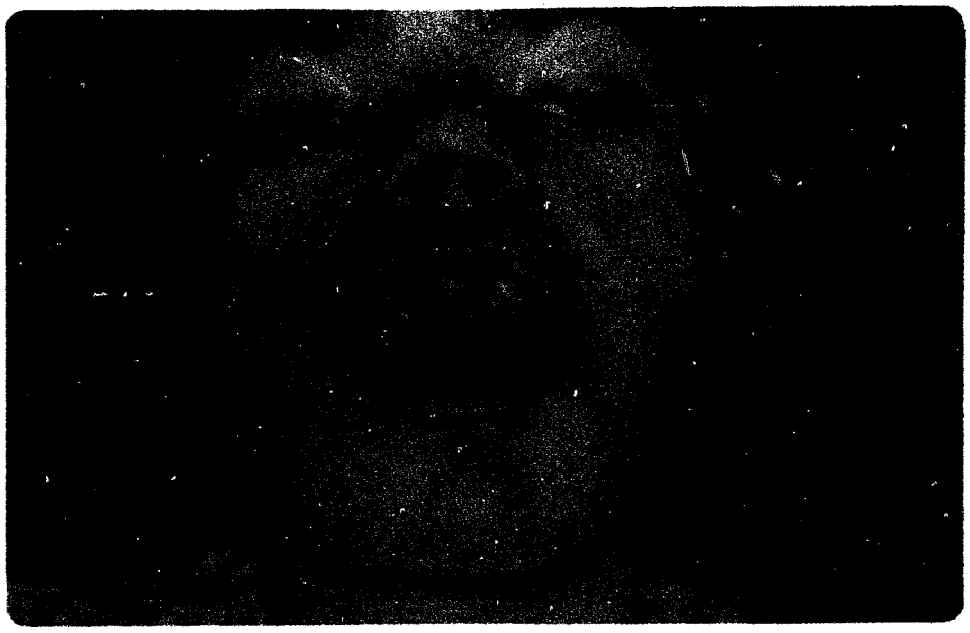


Figure 3.

Intraoral photograph of patient in Figure 2.

Figure 4.

Low-power view of histological section from biopsy specimen (Figure 2) shows a marked increase in depth of the lamina propria. (Hematoxylin and eosin stain. Magnification, X320).

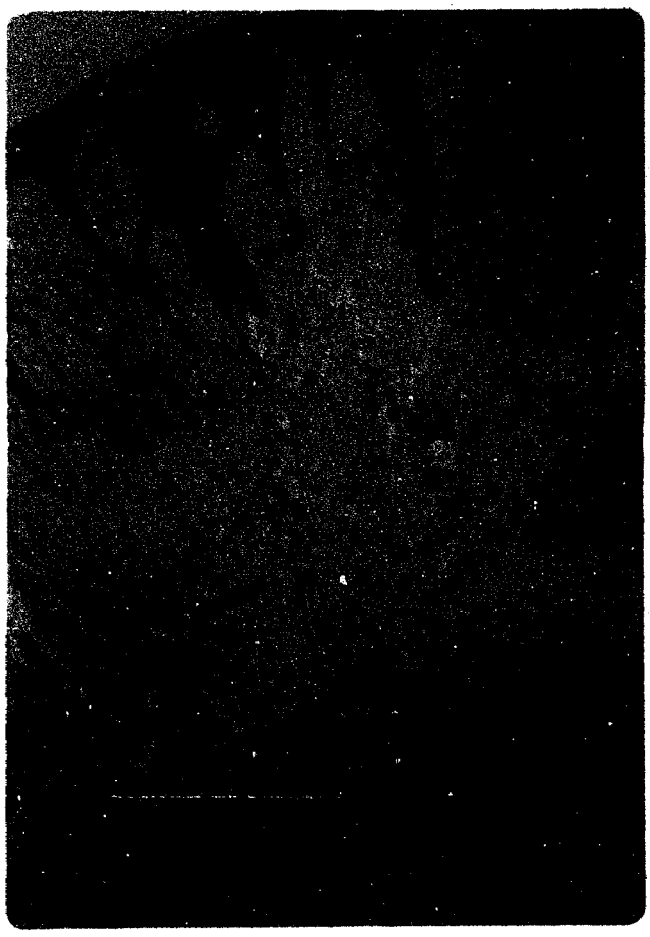


Figure 5.

High-power view of section (Figure 2) shows elongation of the rete ridges and an increased number of vessels in the connective tissue. Note the absence of inflammatory infiltrate. (Hematoxylin and eosin stain. Magnification, X512).

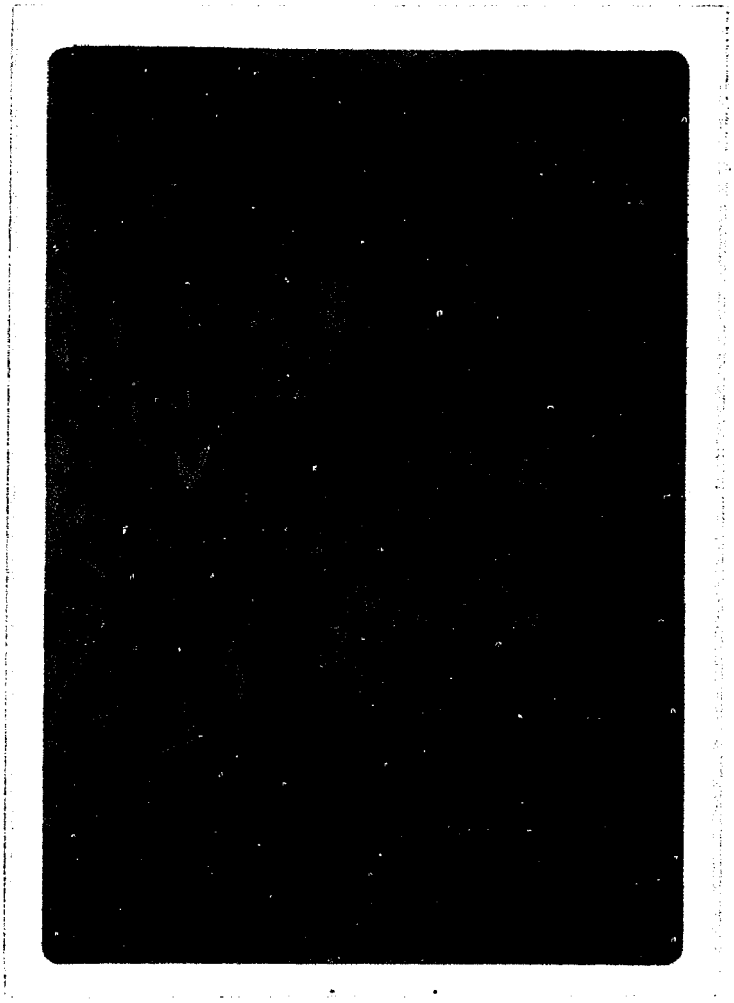


Figure 6.

High-power view of an elongated rete ridge from Figure 5. (Hematoxylin and eosin stain. Magnification, X800)

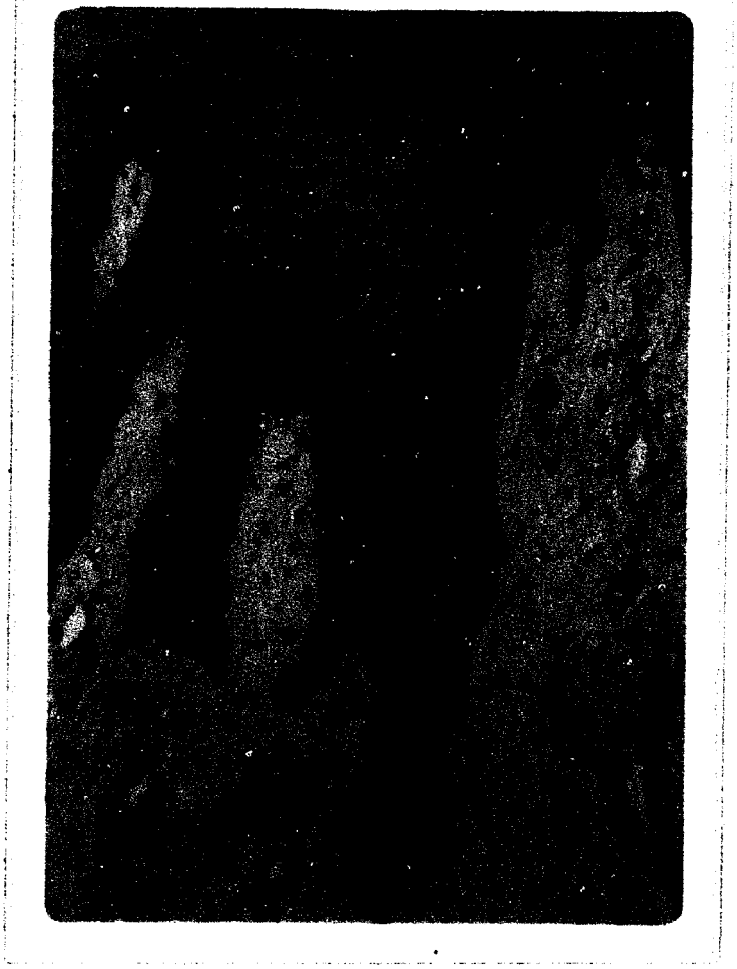


Figure 7.

Oil immersion view of an elongated rete ridge (Figures 4 and 5). Connective tissue cells appear to be mature fibroblasts. Note keratinization within the rete ridge. (Hematoxylin and eosin stain. Magnification, X1280).

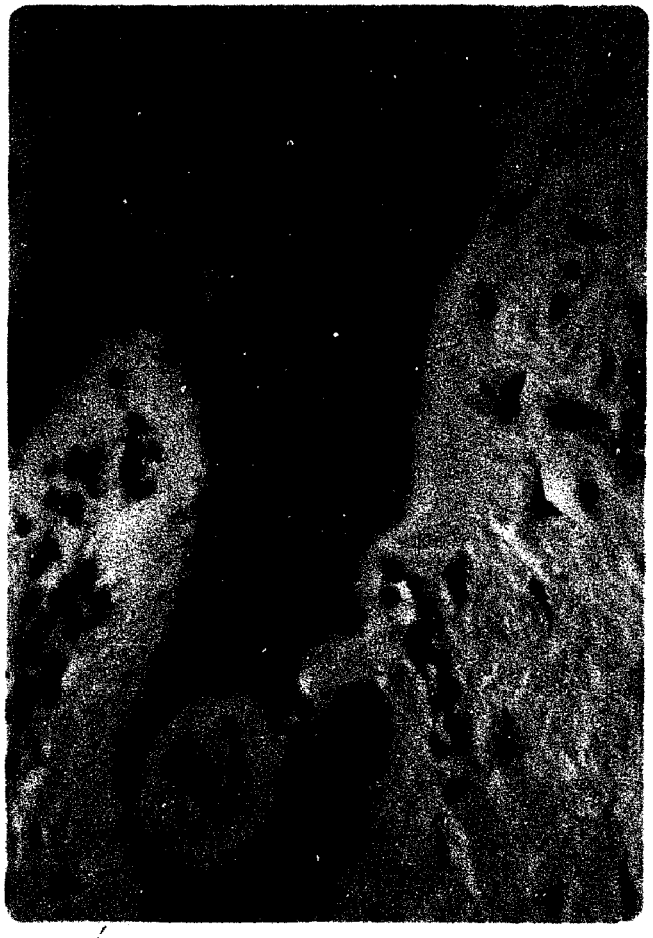


Figure 8.

CM-cellulose elution pattern of CNBr peptides from DPH gingival collagen. 200 mg sample, dissolved in starting buffer (0.02 M sodium citrate, pH 3.6) was eluted with 50 ml of this buffer. The column was then eluted with a linear gradient formed by 1100 ml 0.02 M sodium citrate (pH 3.6) containing 0.02 M NaCl as starting buffer and 1100 ml of 0.02 M sodium citrate (pH 3.6) containing 0.14 M NaCl as limit buffer. The column, maintained at 40° C, eluted at 200 ml/hr.

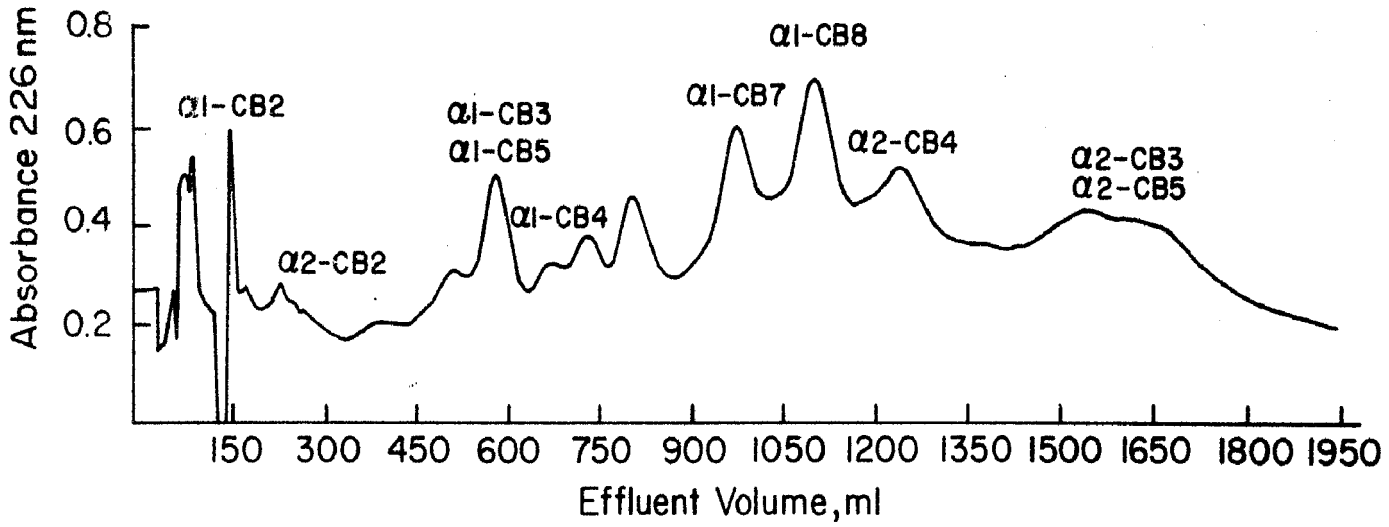

Absorbance 226 nm
0.8
0.6
0.4
0.2
α1-CB2
α2-CB2
α1-CB3
α1-CB5
α1-CB4
α1-CB7
α1-CB8
α2-CB4
α2-CB3
α2-CB5
150 300 450 600 750 900 1050 1200 1350 1500 1650 1800 1950
Effluent Volume, ml

Figure 9.

CM-cellulose elution pattern of CNBr peptides from SI gingival collagen. Chromatographic conditions same as Figure 8.

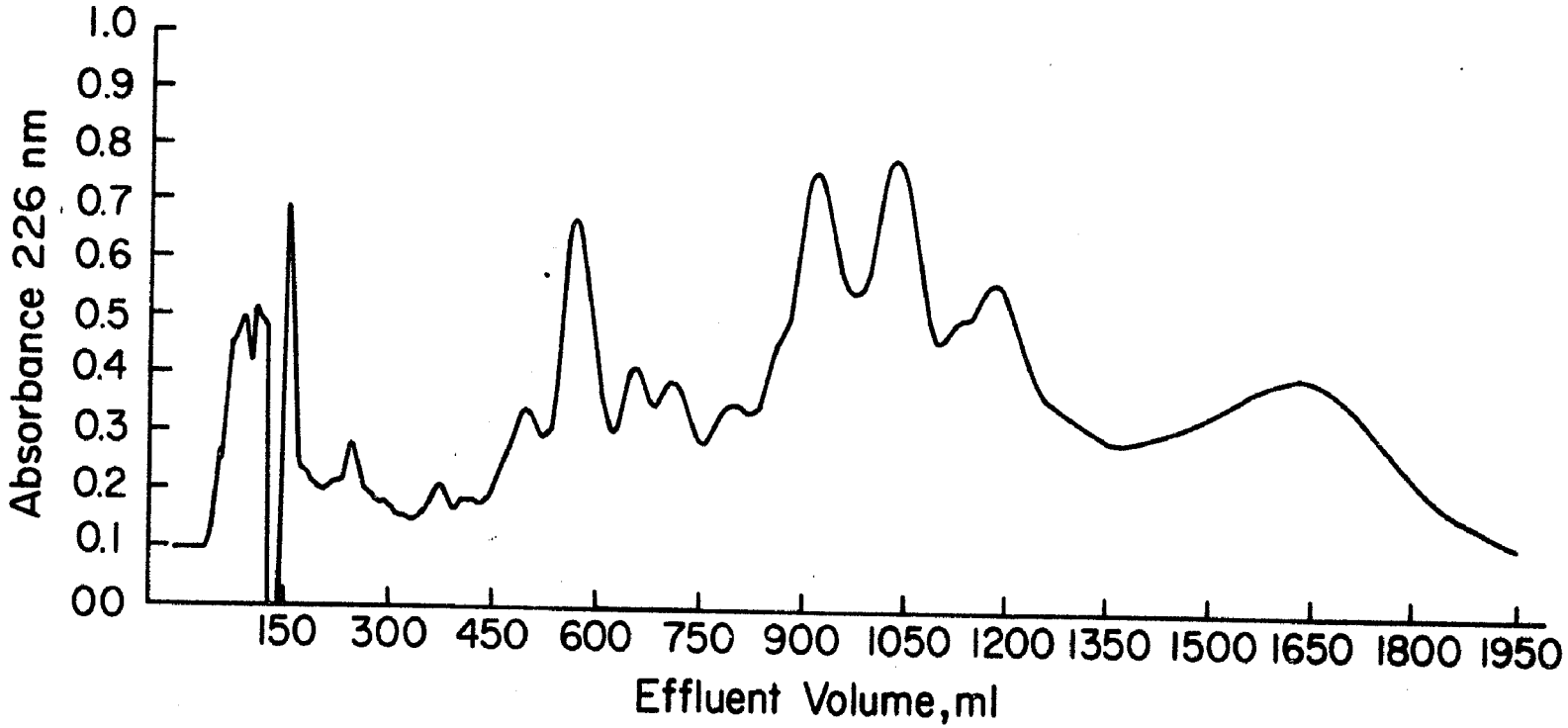

Absorbance 226 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
00
150 300 450 600 750 900 1050 1200 1350 1500 1650 1800 1950
Effluent Volume, ml

Figure 10.

CM-cellulose elution pattern of CNBr peptides from insoluble gingival collagen of patients treated for periodontal disease. Chromatographic conditions same as Figure 8.

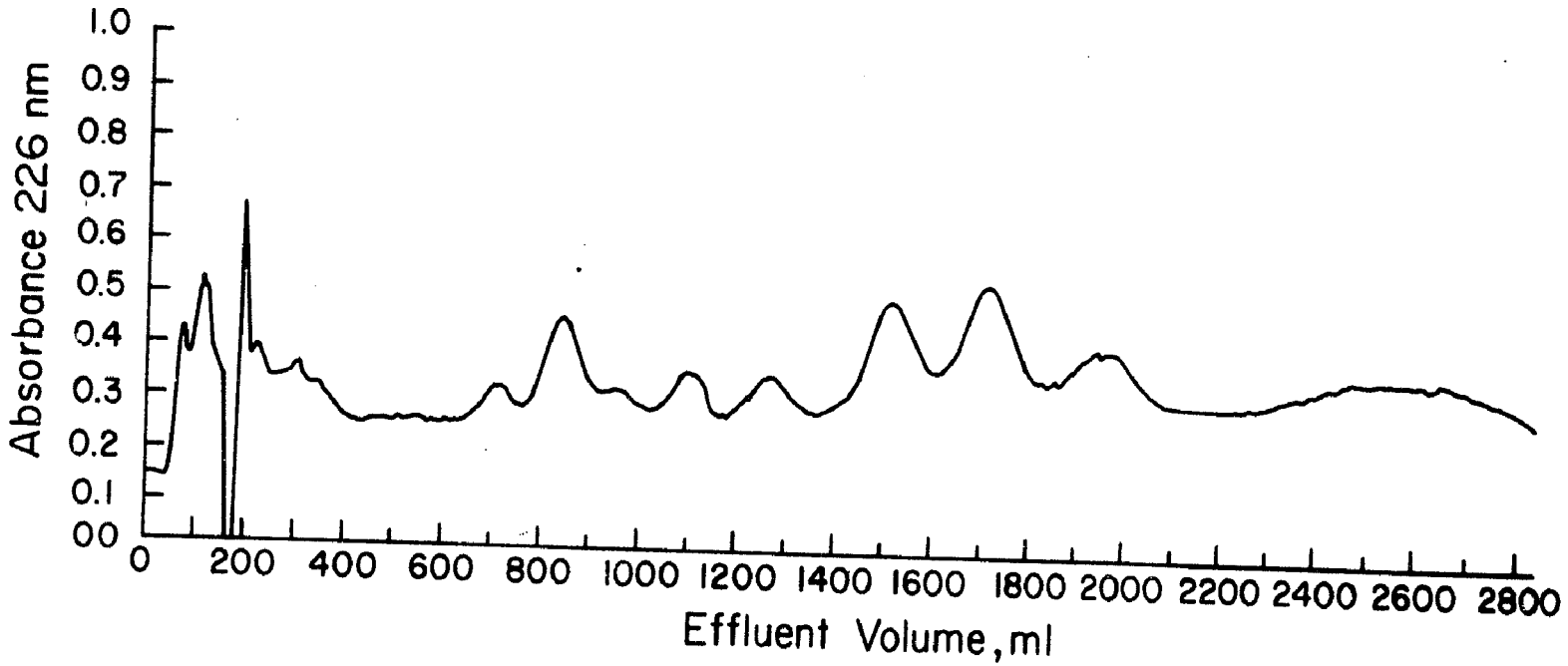

Absorbance 226 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
00
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
Effluent Volume, ml

Figure 11.

The Sephadex G-50 elution pattern of α1-CB3, α1-CB4, and α1-CB5 (the fraction eluted between 450 and 700 ml on CM-cellulose, Figure 1). Samples (5-10 mg) were dissolved in 5.0 ml 0.2 M acetic acid and eluted on a 1.8 x 135 cm Sephadex G-50 column (fine) that was equilibrated with the same solution. Fractions containing 4.4 ml were collected on an LKB Ultrorac 7000 Fraction Collector and absorbance was later read on a Beckman DB-GT spectrophotometer at 226 nm.

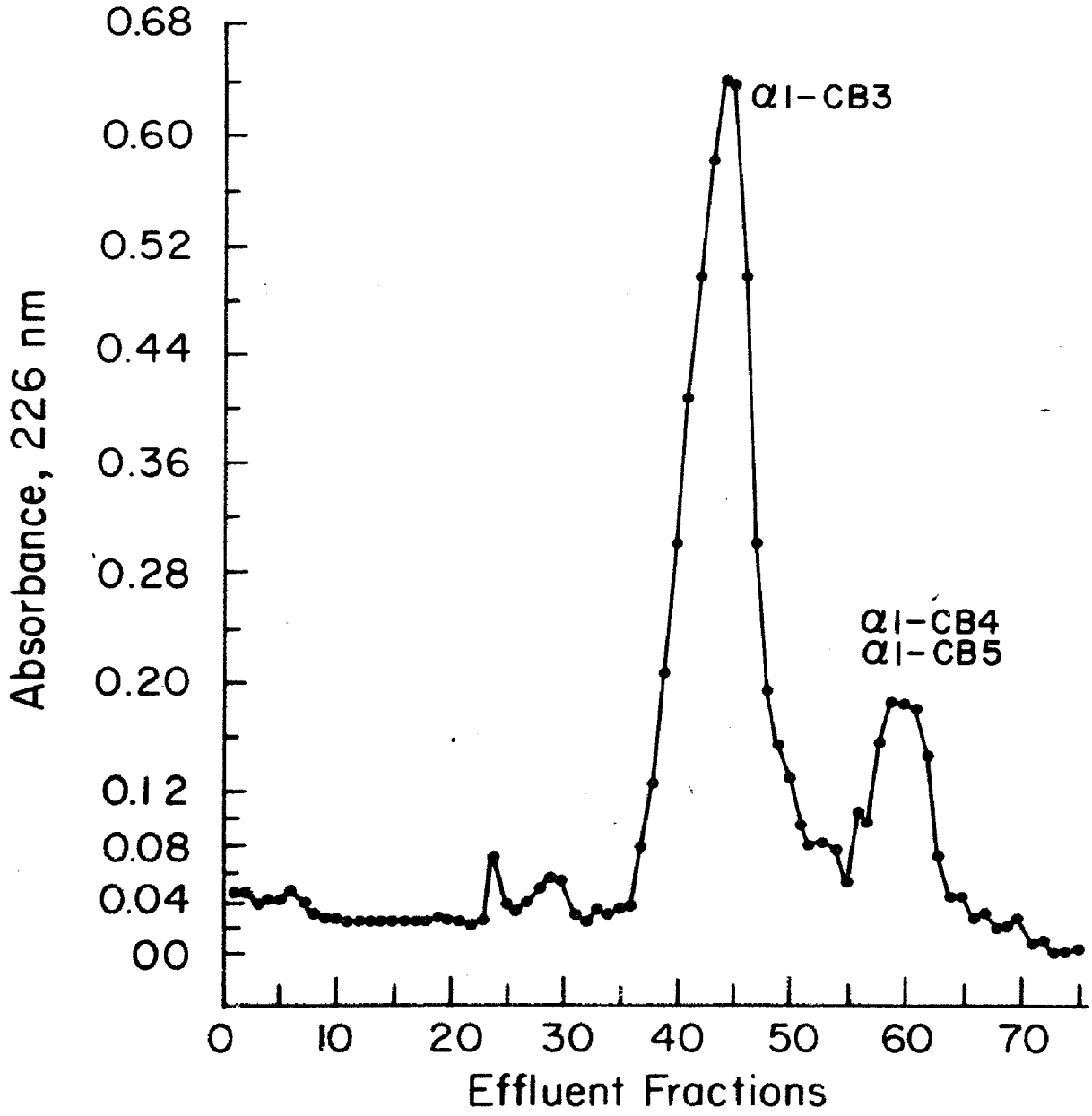
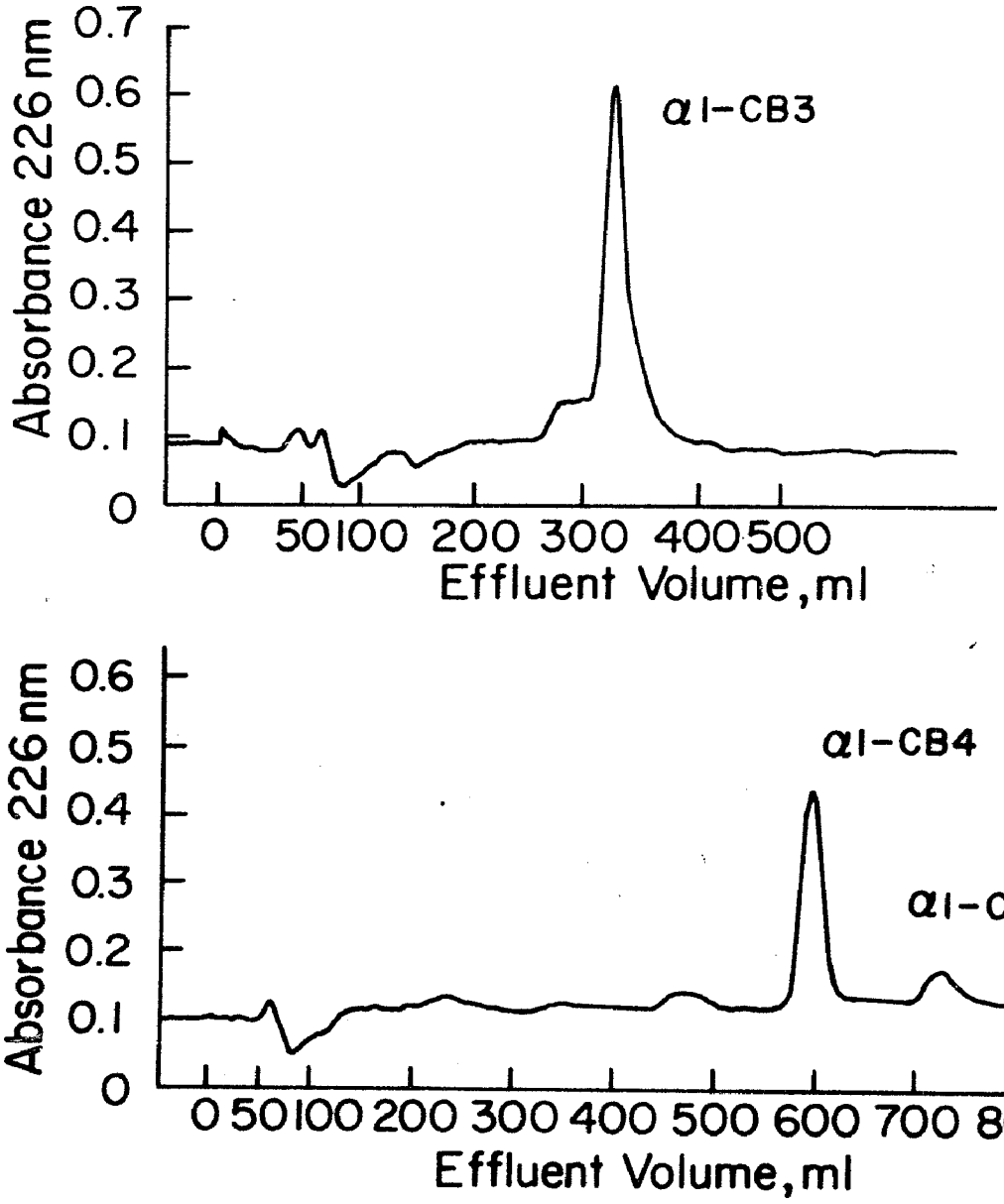

0.68
0.60
0.52
0.44
0.36
0.28
0.20
0.12
0.08
0.04
00
Absorbance, 226 nm
α1-CB3
α1-CB4
α1-CB5
0
10
20
30
40
50
60
70
Effluent Fractions

Figure 12.

Phosphocellulose rechromatography of peaks in Figure 11. Elution patterns obtained on a phosphocellulose column (Whatman, floc, 140-200 mesh) maintained at 40° C. Samples (3 to 5 mg) were dissolved in 5 ml 0.001 M sodium citrate (pH 3.6) and eluted with a linear gradient formed by 500 ml 0.001 M sodium citrate containing 0.1 M NaCl as starting buffer and 500 ml 0.001 M sodium citrate containing 0.8 M NaCl as the limit buffer.


Absorbance 226 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
α1-CB3
0 50100 200 300 400500
Effluent Volume, ml
Absorbance 226 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
α1-CB4
α1-CB5
0 50100 200 300 400 500 600 700 800
Effluent Volume, ml

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Major Subject Biochemistry

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