10 to 13, and with only one residue having a normal value of about 10. The abnormality of these ionizations is certainly a result of their environment; it is reasonable that the residues of altered pK might participate in intramolecular interactions and be responsible in part for stabilizing the native conformation. Although ionization of these groups would be expected to disrupt the folding, the viscosity data show that for the ionization of two and part of the third abnormal tyrosines there is no appreciable change in external shape. However, small changes in *internal* structure may still be associated with the titration of one or more of the tyrosyl groups. This is consistent with data on the iodination of cytochrome *c* (Ishikura *et al.*, 1959). Incorporation of four atoms of iodine led to disruption of certain functional properties of the cytochrome (i.e., it produced inactivity in an oxidase system, autoxidizability, and a capacity to bind carbon monoxide) without appreciable change in heme spectrum, explainable by local conformational changes following iodination (and presumably ionization) of one or more abnormal tyrosine residues.

The stability of the compact molecular structure to *p*H above 13 itself is noteworthy. Proteins generally denature in less alkaline solution, often irreversibly and rapidly. The spectral difference is reversible for cytochrome *c* from *p*H 13.1 (reversibility was not tested at higher *p*H), and at all *p*H values the spectrum did not change over the time of measurement. Previous studies on the guanidine hydrochloride denaturation of cytochrome *c* (W. Kauzmann and J. A. Rupley, unpublished data) have shown that the molecule is unusually stable (8 M urea does not affect the structure), and that conformational changes when they occur are rapid and reverisible. The behavior at alkaline *p*H is in agreement with these properties of the molecule.

Similar experiments on the tyrosyl ionization of cytochrome *c* have been recently reported by Stellwagen (1964), who observed under different experimental conditions (lower salt concentrations) approximately the behavior described. Stellwagen did not analyze the ionizations in terms of dissociation constants, but concluded from other evidence that all four tyrosines were abnormal, in contrast to the single normal ionization inferred from this work.

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The Macromolecular Organization of Dentine Matrix Collagen. I. Characterization of Dentine Collagen*

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Bovine dentine matrix collagen and corium collagen were isolated and purified. These hard- and soft-tissue collagens were then compared in terms of amino acid composition, hexose and hexosamine content, and reactivity of the ϵ-amino groups of lysine and hydroxylysine. The principal differences were that the dentine matrix collagen was found to contain phosphorus (at least partially in the form of phosphate bound to serine as phosphoserine) and that the ϵ-amino groups of lysine were more readily reactive with fluorodinitrobenzene than were similar groups in the corium collagen. Comparative swelling and solubility studies show that the dentine matrix collagen is a highly cross-linked system. Consideration of the potential cross-linking mechanisms leads to the tentative conclusion that the enhanced stabilization of the dentine matrix collagen stems from the presence of phosphate-mediated di- or triester intermolecular cross-linkages.

The major emphasis in research on the basic structure of collagen has been centered on the collagens of the soft tissues because of the relative ease with which these can be purified and, frequently, rendered into soluble form. The collagens which form the principal organic matrices of bones and teeth have not been studied so extensively, although they play an important, if not crucial, role in calcification and tissue stabilization.

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Amino acid analyses (Piez, 1961; Eastoe, 1963) reveal no composition differences of any apparent significance between collagens of the various types, as long as comparisons are restricted to tissues of the same species. The hard- and soft-tissue collagens do appear to differ in their state of physical organization. The protofibrillar elements of the soft-tissue collagens are aggregated in long parallel bundles. In cornea, the collagen fibrils are stacked parallel in layers with the fibril axes in each layer being mutually perpendicular. The bone and dentine matrix collagens, on the other hand, are formed into very fine highly intertwined networks from which individual fibrils cannot be teased. Another difference is in the great difficulty with which soluble components can be extracted from the matrix collagens (Neuman and Neuman, 1958; Saito, 1960; Araya *et al.*, 1961). In view of the probable relationship between soft-tissue-collagen solubility and the presence of intermolecular covalent cross-linkages (Veis *et al.*, 1962), the low solubility of the matrix collagens suggests that these may be cross-linked more highly than the soft-tissue collagens.

Our work on dentine collagen has been undertaken with the aim of discovering the basis for the differences between the hard- and soft-tissue collagens, chemical or physical, and whether these factors intrinsic to collagen could account for the different functions of the two types of tissues. This first paper describes the isolation and purification of bovine dentine collagen, a most intractable material chosen as the epitome of the hard-tissue collagens, and a comparison of its behavior and properties with those of bovine corium collagen.

Experimental

The Collagens.—Bovine corium collagen was isolated from fresh steer hides by the procedure of Veis *et al.* (1960). The fraction utilized in all subsequent analyses is that previously designated as a "purified intact collagen," signifying that repeated acid and slightly basic extractions had removed the soluble collagen components. Phosphate buffers were utilized in the extractions for the neutral salt–soluble fractions. The intact collagen was found to contain 18.0% N, 11.2% hydroxyproline (based on residue weight), and 0.01% phosphate (PO_4).

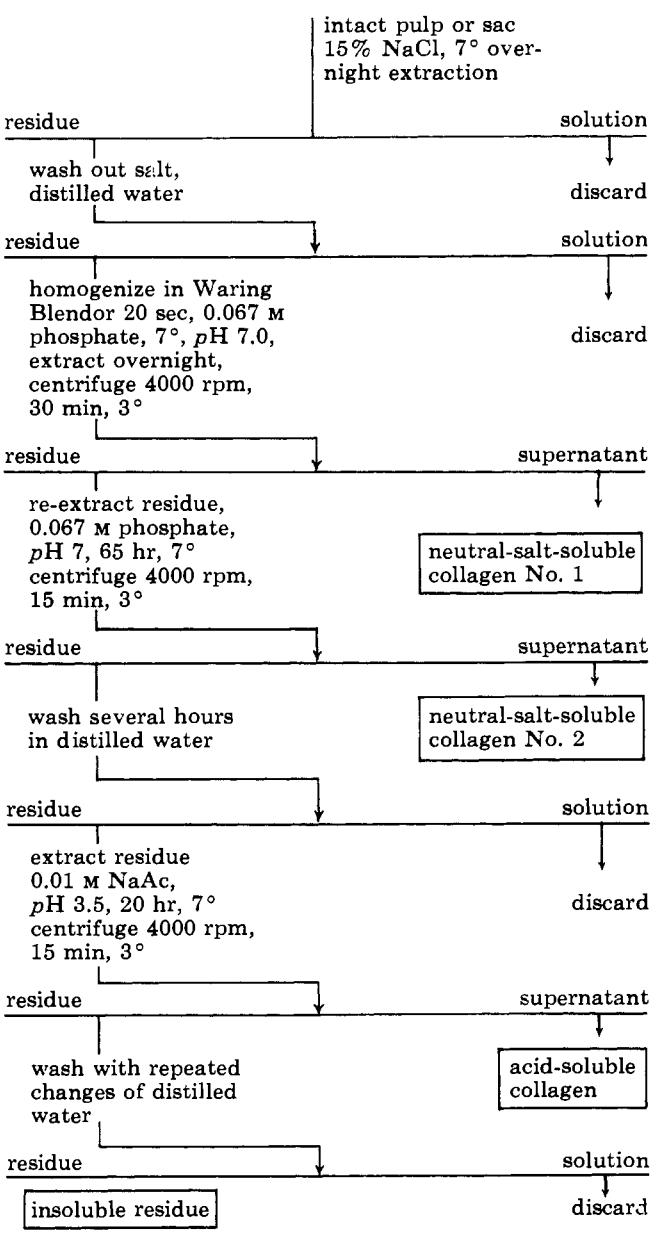
Bovine dentine collagen was isolated from fresh cattle teeth. Fresh cattle jaws were collected and sawed open to expose the unerupted teeth. These unerupted teeth were removed from the jawbone with the sac and pulp still intact. The teeth were rinsed and extracted with ice cold 15% (w/v) sodium chloride solution. After extraction the sac, pulp, and mineralized teeth were separated. From seven jawbones the wet weights of sac, pulp, and mineralized unerupted teeth were 54, 106, and 310 g, respectively.

The teeth were broken into small pieces, slivers about 1–2 cm in length, by crushing with a hammer. These fragments were rinsed in 15% sodium chloride, then washed free of salt with repeated distilled-water rinses. Demineralization was carried out by extraction at 4° with 0.5 M EDTA adjusted to *p*H 7.4 with potassium hydroxide. A ratio of 500 ml of EDTA solution to 300 g wet teeth was used and the suspension was stirred vigorously. The high porosity of the dentine as compared with the enamel (Johansen and Parks, 1962) was of great value in the initial stages. With vigorous agitation the dense but brittle enamel layer flaked off from the dentine core. The enamel fragments and other noncollagen materials formed a turbid suspension which was decanted from the larger dentine pieces. About ten changes of EDTA were used for each preparation, with the same EDTA volume used as for the initial extractions. The EDTA solution was decanted daily during the first several days of extraction but less frequently later. In the last few extractions, half-volumes of EDTA were used. The EDTA extraction was continued until constant values of total nitrogen and phosphorus contents were obtained for the dentine collagen. Analyses were begun when the dentine pieces became flexible and the EDTA extracts became clear. When the EDTA-insoluble residue had constant composition (as judged by nitrogen or phosphorus content), it was washed thoroughly with repeated changes of cold distilled water for several days.

Neutral-salt-soluble and acid-soluble fractions were obtained, in low yield, by 24-hour extractions with *p*H 7, 0.067 M potassium phosphate buffer and *p*H 3.5, 0.01 M acetate buffer, respectively. The intact dentine collagen was washed once again with distilled water and finally was lyophilized. The overall yield of lyophilized intact dentine collagen was 7% of the wet weight of teeth. The purified dentine collagen was found to contain 18.1% N, 12.0% hydroxyproline, and 0.53% phosphate.

The pulp and sac fractions were both treated as indicated in the following flow diagram so that their collagen content might be determined.

Flow Diagram



Chemical Analyses.—AMINO ACID COMPOSITION.—Protein samples were hydrolyzed for 16 hours in 6 N hydrochloric acid at 107° in tubes sealed *in vacuo*. A ratio of 10 mg of collagen to 1 ml hydrochloric acid was used. Amino acid analyses were carried out by the method of Spackman *et al.* (1958) on a Spinco Model 120 amino acid analyzer using 30–50° runs to assure resolution of the hydroxyproline peak.

Phosphoserine is destroyed by the foregoing hydrolysis conditions. When it became evident that phosphoserine might be a constituent of the dentine collagen, a semiquantitative determination of the phophoserine content was made on the analyzer after partial hydrolysis of the protein in 2 N hydrochloric acid *in vacuo* at 107° for 9.5 hours (Kennedy and Smith, 1954).

HEXOSE.—The Helbert and Brown (1956) modification of the anthrone method (Dische, 1955; Ashwell, 1957) was used. Standards were prepared from reagent-grade dextrose in 1 N sulfuric acid. Color yields are expressed in glucose units. The report that glutamic acid gives a positive anthrone test (Ashwell, 1957) could not be substantiated. Maximum color yields were obtained from collagen after 6-hour hydrolysis in 1 N sulfuric acid at 100°. Under these conditions the degradation of glucose was no greater than 6%; appropriate corrections for this degradation were made.

PHOSPHORUS.—The Gomori method (1942) was used. Samples were digested either by dry-ashing at 425° for a minimum of 28 hours in Vycor crucibles, or by heating in sulfuric acid, accelerated by the addition of several drops of 2 N nitric acid or 30% hydrogen peroxide. Care was taken to have the proper acid-molybdate ratio during color development.

CALCIUM.—The procedure of Diel and Ellingboe (1956) and Bett and Fraser (1958), as modified by Jackson *et al.* (1962), was used for precise quantification of the calcium content. This determination is based on the disappearance of a fluorescent calcium-calcein complex upon titration with EDTA in basic media. The same ashing procedure as in the phosphorus determination was used. The ashed samples were dissolved in 1 N HCl.

HYDROXYPROLINE.—In addition to the data obtained by the column-chromatographic method described earlier, hydroxyproline analyses were carried out by a modification of the method of Neuman and Logan (1950). To 2 ml of aqueous hydrolysate containing no more than 25 μg of hydroxyproline was added 2 ml of basic-$CuSO_4$ reagent (equal volumes of 0.01 M $CuSO_4$ and 2.5 N NaOH mixed immediately before use). One ml of 2% H_2O_2 was added and the solutions were shaken gently on a wrist-action shaker for 5 minutes at room temperature. Shaking was then continued vigorously for 10 minutes more to dispel excess H_2O_2. Three ml of 4 N H_2SO_4 was added with mixing and then 2 ml of *p*-dimethylamino benzaldehyde reagent (5% in 1-propanol) was layered on top of each mixture. All tubes were shaken vigorously, heated in a 70° bath for 20 minutes with moderate shaking, and then cooled in an ice bath for 2 minutes. Ten minutes after removal from the ice bath, the optical density of each solution was read at 555 mμ. The colored complex was stable for approximately 1 hour. A standard curve was run for every analysis. The color yield of hydroxyproline was not influenced by the addition of as much as 4×10^{-2} μmoles of ϵ-DNP-lysine.

NITROGEN.—Total nitrogen was determined by a standard micro-Kjeldahl procedure using mercuric sulfate as the catalyst and a 45-minute digestion period (Tristram, 1953).

Preparation of ϵ-DNP-lysine.—The procedure used was that of Porter and Sanger (1948). The α-amino group of lysine was blocked by being complexed with copper, allowing only the ϵ-amino group to react with the FDNB. The copper complex was subsequently broken with H_2S. The crystalline ϵ-DNP-lysine was recrystallized from 20% HCl. The melting-point range was 186–190°. The molar extinction coefficient (1 cm) in 1 N HCl was 1.7×10^4 at 360 mμ, the observed absorption maximum. These values agree well with those reported by Porter (1957), i.e., a melting point of 186° and a molar extinction coefficient of 1.75 $(10)^4$.[1]

Dinitrophenylation of Collagen.—Sanger's technique (1945) was followed. In addition to reacting the corium collagen in 66% (v/v) ethanol, the reaction was also carried out in 100% ethanol both with and without added $NaHCO_3$. No differences in yield were noted. The DNP-collagens were hydrolyzed (with appropriate controls) for 16 hours in 6 N HCl at 107°. The hydrolysates were extracted with ether, concentrated, and then diluted to an appropriate volume with water. The equivalent protein concentrations of the dilutions were determined both by total nitrogen and by hydroxyproline analysis. The concentration of the ϵ-DNP-lysine was determined from the extinction coefficient. It was assumed that ϵ-DNP-hydroxylysine had the same molar extinction coefficient as the ϵ-DNP-lysine.

Control experiments showed 100% recovery of ϵ-DNP-lysine when this compound was hydrolyzed with steer-hide corium collagen and 96.5% when hydrolyzed with dentine collagen. Appropriate corrections have been made for the breakdown in the dentine collagen data reported (*vide infra*).

Collagenase Digestion.—A Nutritional Biochemicals Corp. preparation of collagenase, lot 8860, was used under the conditions described by Mandl *et al.* (1953), i.e., 0.067 M phosphate buffer at *p*H 7.4, containing 0.45% NaCl. Digestions were carried out at room temperature, using a substrate-enzyme weight ratio of 100:1. Only intact or insoluble native collagen samples were used as substrates.

Thermal Solubilization.—Collagen samples were suspended in glass-distilled water with a 100:1 water to protein (v/w) ratio. The suspensions were heated in appropriately thermostated baths and aliquots were withdrawn at various time intervals. The gelatin concentrations of these aliquots were determined by the Biuret method (Layne, 1957).

Swelling.—The dentine and corium collagen pieces were cut into approximately 2-mm cubes. These were soaked in a large volume of glass-distilled water. After thorough wetting, and using several changes of water, the cubes were distributed equally into graduated conical centrifuge tubes and suspended in 10 ml glass-distilled water. The tubes were centrifuged for 2 minutes at 2000 rpm and the packed volume, on the order of 1 ml, was recorded. The water supernatant was decanted and replaced with hydrochloric acid solutions of varying *p*H. After several changes of the acid solutions the cubes were left suspended and the tubes were stored at 4–5° for 24 hours. The tubes were then warmed to room temperature and recentrifuged. The final packed volume of the collagen and the equilibrium *p*H of the supernatant were recorded. The ratio of the volume increase to the initial packed volume was calculated as the per cent swelling, although this method does not take into account the void spaces.

[1] This extinction coefficient has been estimated from Porter's spectral-transmission curve for ϵ-DNP-L-lysine.

TABLE I

YIELDS OF COLLAGEN COMPONENTS FROM DENTINE AND RELATED TISSUES EXTRACTED FROM UNERUPTED BOVINE TEETH

Tissue Extracted	Fraction[a]	Yield (% dry basis)	Nitrogen (%)	Hydroxy-proline (%)	Hydroxy-proline/ Nitrogen
Decalcified dentine	NSS-2	0.09	8.95	0.26	0.029
	AS	0.01			
	Insoluble	99.80	17.60	12.4	0.705
Sac	NSS-1-A	0.57	7.9	0.2	0.025
	-B	1.60	12.6	1.96	0.156
	-C	7.34	9.2	0.19	0.20
	NSS-2-A	0.74	10.9	0.18	0.016
	-B	0.96	11.9	1.88	0.158
	-C	0.91	13.1	0.14	0.011
	AS -B	0.12	17.3	11.1	0.642
	-C	0.41	7.4	5.35	0.726
	Insoluble	87.35	16.2	9.9	0.610
Pulp	NSS-1-A	0.24	11.1	0.25	0.023
	-B	0.92	12.8	2.71	0.212
	-C	17.02	13.3	0.20	0.015
	NSS-2-A	0.64	12.5	0.09	0.007
	-B	1.19	15.3	1.72	0.112
	-C	1.85	12.5	0.10	0.008
	AS -B	0.05			
	-C	0.14	1.8	0.09	0.05
	Insoluble	77.95	14.5	7.6	0.524

[a] NSS, neutral-salt-soluble fraction; AS, acid-soluble fraction; A, precipitate upon dialysis against 1% NaCl; B, precipitate upon dialysis against distilled water; C, soluble in distilled water.

TABLE II

AMINO ACID COMPOSITION OF HARD AND SOFT TISSUE COLLAGENS

	Steer Corium		Cow Corium		Bovine Dentine (unerupted)		Bovine Dentine[a]	Human Dentine (unerupted)[b]
	Weight (%)	Residues/ 1000	Weight (%)	Residues/ 1000	Weight (%)	Residues/ 1000	Residues/ 1000	Residues/ 1000
Lysine	3.40	24.8	3.44	24.1	2.60	18.9	22	25
Hydroxylysine	0.81	5.2	0.94	5.8	1.43	9.2	9.6	8.1
Histidine	0.70	4.8	0.88	5.8	0.77	5.2	4.7	6.5
Arginine	7.98	47.9	8.47	48.7	7.78	46.9	52	51
Glutamic acid	9.93	72.1	10.82	75.3	9.85	70.9	74	75
Aspartic acid	5.81	47.3	5.82	45.5	6.18	49.9	46	51
Threonine	1.79	16.6	1.81	16.1	1.85	17.0	17	20
Serine	3.64	39.2	3.37	34.8	3.58	38.2	33	41
Hydroxyproline	11.36	94.1	11.16	88.7	12.02	98.8	99	98
Proline	13.36	129.0	13.29	123.0	12.32	118.0	116	115
Glycine	20.51	336.5	21.83	343.4	20.04	326.3	329	308
Alanine	8.09	106.6	9.14	115.5	9.51	124.9	112	108
Valine	2.06	19.5	2.09	19.0	2.21	20.7	25	27
Methionine	0.55	3.9	0.55	3.8	0.54	3.8	5.3	6.8
Cysteine	0.00	0.0	0.00	0.0	0.00	0.0		
Leucine	2.89	24.0	2.97	23.5	3.01	24.7	24	27
Isoleucine	1.37	11.3	1.44	11.4	1.36	11.2	9.3	12
Tyrosine	0.80	4.6	0.72	4.0	0.70	4.0	6.4	4.9
Phenylalanine	1.97	12.6	1.94	11.9	1.88	11.9	16	15
Ammonia	0.74	(41.8)	0.78	(41.9)	0.83	(37.3)	(38)	(54)
Glucosamine	0.00	0.0	0.00	0.0	0.00	0.0		
Hexose[c]	0.39	(2.1)	0.40	2.1	0.40	(2.2)		
Phosphate	0.02	(0.2)		(—)	0.53	(5.8)		
Nitrogen	18.0		18.0		18.1			

[a] Piez (1961). [b] Eastoe (1963). [c] Measured as anthrone-glucose units.

RESULTS

A. *Chemical Characterization.*—The results of the preparative isolations of the collagen components from the dentine matrix, sac, and pulp are listed in Table I. The "yield" column is the total dry-basis fractional-weight recovery. The hydroxyproline-nitrogen ratio in the last column is indicative of the collagen content. In mammalian collagens this ratio has values in the range 0.70–0.74. It is clear that the dentine matrix yielded virtually no acid-soluble or neutral-salt-soluble collagens, whereas the insoluble residue was essentially all collagen. The sac and pulp likewise yielded very little neutral-salt-soluble collagen, although other nitrogenous components were extracted. Acid-soluble collagen was present in the sac, whereas this component was absent from the pulp. Both sac and pulp had large insoluble residues which were composed of about 80% collagen.

The amino acid compositions of bovine corium collagen and dentine matrix collagen are compared in Table

TABLE III
CALCIUM AND PHOSPHORUS CONTENT OF DENTINE AND CORIUM COLLAGENS

Material Tested	Calcium (%)	Phosphorus (%)	Calcium/ Phosphorus
Steer corium, purified	0.31×10^{-3}	5.64×10^{-3}	0.056
Dentine matrix, EDTA extracted	0.82×10^{-3}	0.38	0.002
Undecalcified bovine dentine	33.69	15.91	2.12
$Ca_3(PO_4)_2$	37.11	18.56	2.00
$Ca_3(PO_4)_2$ (calcd)	38.76	19.97	1.94
Calcium hydroxyapatite (calcd) $[Ca_3(PO_4)_2]_3Ca(OH)_2$	39.89	18.50	2.16

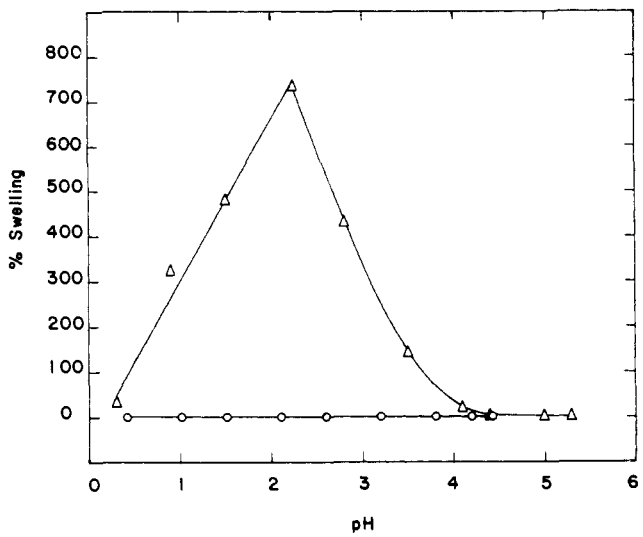


FIG. 1.—Swelling of collagens in acid solutions in salt-free systems. Δ, native corium collagen; O, native dentine collagen.

II, along with some data of others. It can be seen that, as far as the amino acid moieties are concerned, there are no striking differences in composition between the dentine and corium collagens. However, each analysis is sufficiently different so that one may not rule out subtle variations in the details of sequential arrangements. The outstanding feature of the analyses reported here is the presence of phosphate in relatively large amounts in the thoroughly decalcified dentine. The trace amounts of phosphate found in the corium collagen is believed to represent adventitious phosphate bound during the phosphate buffer extraction for the salt-soluble collagens. No special precautions were taken, as in the case of the dentine, to remove this phosphate. To verify that this was not the source of the dentine collagen phosphate, several phosphorus analyses were carried out on the dentine collagen immediately after EDTA extraction and before phosphate buffer extraction for the soluble collagens. These samples were washed only with distilled water to remove the EDTA. The phosphorus content of this collagen was identical with the phosphorus content reported in Table II.

A possible source of the phosphate might have been trace amounts of calcium hydroxyapatite remaining in the matrix even after the exhaustive EDTA extraction. Analyses of the Ca/P ratio, Table III, eliminate this possibility.

Having eliminated exogenous phosphate, the next obvious source of the phosphate was endogenous phosphate bound to serine or one of the other hydroxyamino acids by an ester linkage. Partial acid hydrolysates of the dentine collagen clearly showed the presence of a peak corresponding to the phosphoserine position when subjected to column chromatography. Similar treatment of the corium collagens did not give rise to any material in this position. In view of the similarity in composition of the two collagens in other respects than phosphate content, the material in the phosphoserine peak position is thought to be phosphoserine. The fact that phosphoserine is almost completely hydrolyzed under the more usual drastic hydrolysis conditions probably explains why Piez (1961) and Eastoe (1963) did not observe the presence of this compound in their dentine collagen hydrolysates.

TABLE IV
AVAILABILITY OF ε-AMINO GROUPS TO REACTION WITH FLUORODINITROBENZENE

Source of Lysine Content Data	Collagen Type	Lysine (%)[a]	Hydroxylysine (%)[a]	DNP Reacted[b] (%)
This paper	Dentine	2.60	1.43	89.5
Hess and Lee (1954)	Dentine	3.34	1.0	91.6
Piez (1961)	Dentine	3.30	1.62	82.4
This paper	Steerhide	3.40	0.81	71.0
Bowes *et al.* (1957)	Oxhide	4.00	1.2	70.6
Tristram (1953)	Oxhide	4.47	1.1	66.0

[a] These values in this paper have been calculated using the residue weight of the amino acids for the conversion of moles to grams. The cited literature values have apparently used amino acid weight (i.e., residue weight plus water) for the same conversion. [b] These values have been calculated using the ε-DNP-lysine content determined in the present work and the analytical lysine content data from the sources listed.

Solomons and Irving (1958) reported that the ε-amino groups of demineralized dentine were 96–100% reactive to FDNB, whereas the ε-amino groups of native soft-tissue collagens were only 64–70% reactive. Solomons and Irving relied on the lysine and hydroxylysine content values reported by Hess and Lee (1954) and Bowes *et al.* (1957) for their comparison. FDNB reactivity data are itemized in Table IV. It is evident that there is a distinctly greater availability of the dentine collagen ε-amino groups as compared with those in the corium collagen. However, our data do not entirely confirm those of Solomons and Irving (1958) in that 10–18% of the dentine ε-amino groups (corresponding to 3–5 ε-amino residues/1000 residues) were blocked from FDNB reaction.

Exposure of the dentine and corium collagens to the action of collagenase led to the eventual complete solution of both materials. Solubilization of the corium collagen was achieved about twice as rapidly as for the dentine matrix collagen.

B. *Physical Characterization.*—The degree to which an insoluble network gel can be swollen is directly related to the extent of cross-linkage of the structure (Flory, 1953). The swelling behavior of dentine matrix collagen compared with that of bovine corium collagen as a function of *p*H in salt-free solutions is

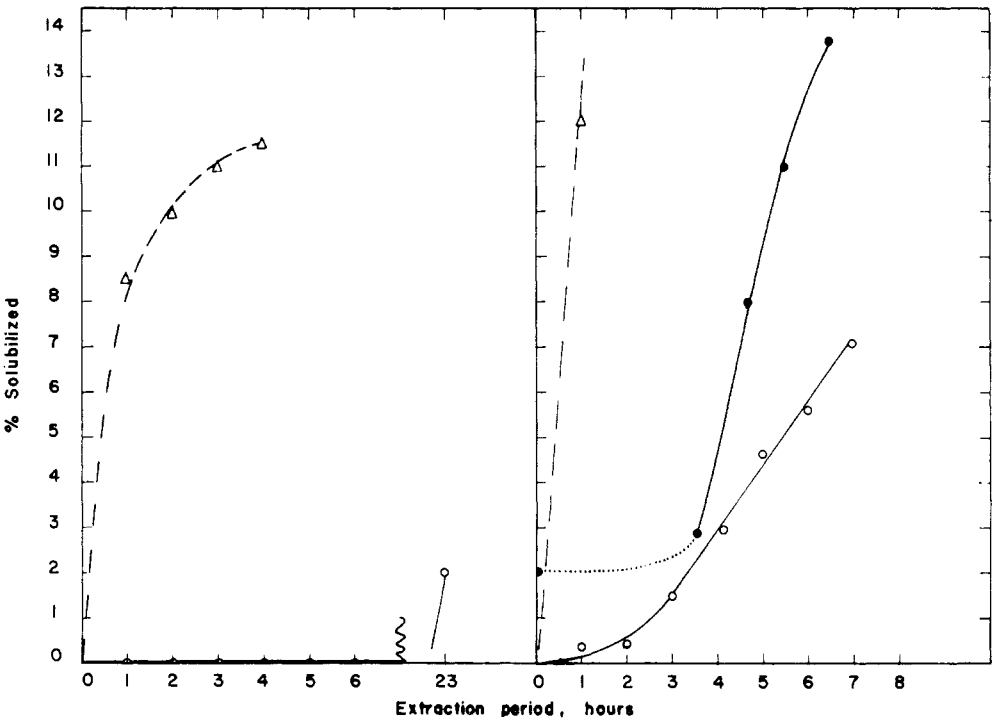


FIG. 2.—Solubilization of collagen under isoionic conditions. Left-hand plot, extraction at 60°. Right-hand plot, extraction at 80°. △, native corium collagen; ○, native dentine collagen; ●, dentine collagen extracted at 60° for 23 hours, then extracted at 80° for the indicated period.

depicted in Figure 1. The dentine collagen was found not to swell at all over this *p*H range, in which the corium collagen swelling was so enormous. Even after the tubes containing the dentine collagen were allowed to stand at room temperature for an additional 2 weeks, no swelling could be discerned and the dentine collagen pieces remained opaque. After only 1 day the swollen corium collagen was translucent. Additional swelling measurements were carried out at *p*H 2.3, the value for maximum corium swelling, in the presence of 8.0 M urea. The corium pieces were completely disaggregated by this treatment but the dentine collagen appeared to be virtually unaffected.

Another view of the integrity of a polymerized network can be obtained by studying the solubilization of the network. As anticipated from the swelling data, the dentine matrix was much less soluble than the corium collagen under comparable conditions (Fig. 2a,b). Extraction at 60° for 6 hours resulted in the solubilization of barely detectable trace amounts of dentine, whereas isoionic extraction of the corium yielded 8.5% soluble collagen in the first hour. After 23 hours at 60° about 2% of the dentine collagen had been extracted as gelatin (Fig. 2a).

Extractions at 80°, where peptide-bond hydrolysis proceeds much more rapidly than at 60°, resulted in the solubilization of substantial amounts of the dentine collagen, although again the corium collagen solubilized to a much greater extent much more rapidly (Fig. 2b). The dentine-collagen 80° solubilization, in contrast to the corium extractions, was characterized by a definite lag or induction period. This was also strikingly evident in the 80° extraction of the residual insoluble dentine extracted at 60° for 23 hours. After the induction period, during which it appears that about 2% of the collagen is solubilized, the rate of solution or conversion to gelatin is more rapid for the 60° pretreated dentine matrix.

DISCUSSION

Microscopic examination of stained sections of dentine matrix collagen shows the structure to be composed of a three-dimensional network of fine fibrils, in contrast to thicker and less highly entwined fibrils in corium or tendon collagen. The resistance of the dentine matrix to acid swelling may well arise, in part, from the extensive mechanical entanglement in the matrix collagen. However, it is more likely that the resistance to swelling is a manifestation of a high degree of polymerization of the collagen matrix. The solubility data lead to the same conclusion on two counts. First, an appreciable solubilization of the dentine could be accomplished only under conditions of temperature, *p*H, and time conducive to hydrolysis of the peptide bonds of the backbone peptide chains. In the case of the 80° solubilization of the dentine collagen pretreated at 60°, the rate of solubilization was approximately double that of the collagen extracted at 80° without pretreatment. This is the type of behavior predicted by Veis and Cohen (1956) for the depolymerization of network structures in which long chains were joined by a number of transverse cross-linkages. Another feature of such systems is that more drastic treatment can lead to the appearance of higher-molecular-weight network fragments. This was borne out in the present study by the observation that 5-hour-soluble gelatin from the 60–80° extraction had a higher sedimentation coefficient than that from the direct 5-hour 80° extraction.

A second observation clearly indicating the complete polymerization of the dentine collagen matrix is the total absence of both neutral-salt-soluble and acid-soluble tropocollagens. Since the teeth examined were developing, unerupted teeth, it should have been possible to extract a substantial neutral-salt-soluble fraction if it were present. When no neutral-salt-soluble fraction was obtained, it was thought that this component might have been extracted in the EDTA solutions used for demineralization. These EDTA solutions were analyzed for their collagen content but no collagen was found. (An interesting noncollagenous protein-carbohydrate complex to be described elsewhere was isolated from the EDTA.) It thus appears

that if the dentine matrix is formed from a neutral-salt-soluble type biological-precursor collagen, as are the soft-tissue collagens, a particularly active maturing or cross-linking mechanism is involved.

From the point of view of composition, on the other hand, there are very few noteworthy differences between the dentine matrix and corium collagens. Except for the presence of phosphate in the dentine matrix, there are no differences in the amino acid content of the dentine and corium collagens larger than the differences found in various soft-tissue collagens of different origin. Both collagen preparations examined were free of hexosamine and each contained a comparable quantity of hexose and of amide nitrogen.

So far, based on studies of the soft-tissue collagens, three types of covalent interchain cross-linkages have been proposed. Mechanic and Levy (1959) showed the presence of glutamyl-γ-carboxyllysyl-ϵ-amino amide bonds in steer corium collagen and suggested that these bonds might serve as interchain junction points. After demonstrating the presence of bonds in collagen which were susceptible to hydroxylamine and hydrazine, Gallop *et al.* (1959) and Blumenfeld and Gallop (1962) proposed that six intrachain esterlike bonds joined four basic subunit peptide strands of collagen (mw ~25,000) together into the α-units of collagen (mw ~100,000), and further proposed that transesterification reactions might lead to the formation of interchain bonds. Hörmann *et al.* (1961), also from studies of the hydroxylamine reactivity of collagen, plus analyses of the hexose content, proposed that the esterlike linkages involved either one or two hydroxyl groups of the hexoses, plus a glycoside bond. Such linkages could be either intra- or intermolecular. Blumenfeld *et al.* (1963) have shown, however, that while the glucose and galactose of ichthyocol are attached to the protein through a glycosidic bond, the hydroxyl groups at positions 2, 3, 4, and 6 are unsubstituted. Hence, the glucose and galactose moieties present in ichthyocol do not appear to participate in covalent cross-linking. Landucci *et al.* (1958) reported that aldehydes such as methyl glyoxal were present in all collagenous tissues and that these might serve as cross-linking agents. Gallop *et al.* (1964) also suggested that an aldehyde or aldehyde derivative might serve as the cross-linking agent and Milch (1963) showed that a few aldehyde intermediates in normal metabolic pathways were capable of cross-linking collagen. Veis and Drake (1963) demonstrated that monofunctional aldehydes could form both intramolecular and intermolecular cross-linkages in ichthyocol collagen.

The ϵ-amino group reactivity data, which show that the ϵ-amino groups of dentine matrix collagen are blocked to a much lesser extent than the corresponding groups in corium collagen, indicate that extensive cross-linking of the γ-glutamyl carboxyllysyl-ϵ-amino amide type is unlikely. However, some three to five amide-type cross-linkages per 1000 residues, or about nine amide cross-linkages per tropocollagen molecule cannot be excluded.

The hexose content of the dentine collagen, as measured in anthrone-glucose equivalents (2.2 residues/1000) is, within experimental error, the same as in the corium collagen, and in ichthyocol (Blumenfeld *et al.*, 1963). In ichthyocol Blumenfeld and co-workers determined that the glucose-anthrone color yield was approximately twice the galactose color yield, and that there was twice as much galactose (i.e., 1.2 residues glucose, 2.2 residues galactose for 3.2 residues hexose per 1000 amino acid residues). Since the equivalent number of hexoses in ichthyocol do not appear to be involved in cross-linking, a like situation probably exists in the dentine collagen; or, at least, it is likely that hexose would be involved to the same extent in the dentine and corium collagen systems. In the same vein, Landucci *et al.* (1958) have demonstrated that ossein and skin collagens have nearly the same content of "aldehydes" reactive with 2-thiobarbituric acid. Without elimination of aldehyde-mediated cross-linking in dentine matrix collagen we can as a first approximation rule out such bonds as a source of the enhanced stabilization of the dentine matrix.

The above considerations, each suggesting that cross-linking mechanisms responsible for the maturation of the soft-tissue collagen cannot account for the enhanced stabilization of the dentine matrix collagen, leads us to a tentative conclusion that the phosphate moieties of the matrix collagen are involved in the matrix stabilization. This statement is not intended to imply that amide-, ester-, and aldehyde-mediated cross-linkages are not present in the dentine collagen. Rather, we propose that phosphate di- or triester bonds provide an additional network of intermolecular cross-linkages in the dentine collagen.

The next papers in this series describe experiments designed to examine the validity of the conclusions described above regarding, particularly, the questions of carbohydrate-mediated ester linkages and the nature of the phosphate in the dentine matrix collagen.

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The Macromolecular Organization of Dentine Matrix Collagen. II. Periodate Degradation and Carbohydrate Cross-Linking*

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Dentine and corium collagens were subjected to thermal hydrolyses and to periodate oxidation in an attempt to isolate intact chain sequences containing the dentine-collagen cross-link sites. The action of periodates on both dentine and corium was found to be identical, with an identical rate and extent of solubilization. The corium collagen yielded two high-molecular-weight soluble fractions, one of which was insoluble in acid. The soluble dentine fraction contained at least two electrophoretically distinct components, one with a mobility at *p*H 5.3 of -14×10^{-5} cm²/v-sec. These soluble dentine fractions were very rich in nondialyzable phosphate, serine, aspartic acid, and glutamic acid. The relative phosphate content of the insoluble residue was reduced. Periodate oxidation completely destroyed all the tyrosine in each of the systems. Arginine and histidine appeared enhanced in the dialyzable peptides of low molecular weight. It is suggested that the corium and dentine collagens contain a common set of intermolecular cross-linkages, involving the tyrosine-rich regions of the collagen-monomer units, and that the dentine collagen contains, in addition, a set of phosphate cross-linkages distributed in specific regions of high charge density along the body of the monomer units. About 60% of the phosphate esters may be involved in diester cross-link formation. Some carbohydrate cross-linking is also indicated in the dentine system.

In the first paper in this series (Veis and Schlueter, 1964) it was shown that dentine matrix collagen was highly resistant to thermal solubilization and that this was probably owing to the presence of a network of intermolecular cross-linkages not found in the soft-tissue collagens. These cross-linkages were ascribed to the presence of phosphate moieties as integral parts of the dentine-matrix structure.

Grassmann and Kühn (1955) reported that collagen could be solubilized by reaction with periodate in neutral solutions and Hörmann and Fries (1958) proposed that periodate oxidation occurred specifically at the cross-linkages in (soft-tissue) collagen rather than at peptide bonds in the backbone chains. Hörmann and Fries (1958) found no evidence for peptide chain breakage in collagen after reacting it with periodate in 10% acetic acid at 40° for 72 hours or at 20° for 250 hours. We attempted to make use of the periodate oxidation of dentine matrix collagen to render it into soluble fragments with the postulated phosphate bonds intact. The periodate oxidation reactions in dentine collagen did not appear to follow the course outlined by either Grassmann and Kühn (1955) or Hörmann and Fries (1958). However, the principal aim was accomplished; the collagen was partially solubilized and phosphate-rich chain fragments were isolated. This paper describes the periodate oxidation of the dentine matrix collagen and the insight this provides as to the possible nature of both the phosphate and carbohydrate linkages in the intact structure.

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EXPERIMENTAL

The isolation, purification, and properties of the bovine dentine matrix collagen and intact bovine corium collagen are described in detail in the first paper of this series (Veis and Schlueter, 1964). The methods for amino acid analysis, hydroxyproline content, nitrogen content, biuret assay, hexose content, phosphorus content, thermal solubilization, and swelling ability are also detailed in paper I.

Sodium Periodate–Solubilization Reactions.—The reaction conditions employed by Grassmann and Kühn (1955) were used with only moderate modifications. The insoluble-collagen samples, in the form of small cubes, were suspended in a solution of 0.025 M sodium (meta) periodate and 0.025 M sodium bicarbonate, adjusted to *p*H 7.75 with concentrated sodium hydroxide prior to the addition of the collagen. One hundred ml of the buffered periodate solution was used per 1 g of collagen. The reaction flasks were stoppered and wrapped in aluminum foil to keep the contents dark. They were then immersed in a 40° water bath and shaken gently. At the end of the reaction period, normally 30 hours, the suspensions were filtered through coarse sintered glass. The *p*H of the filtrate rose about