Neuman, W. F., and Neuman, M. W. (1958), The Chemical Dynamics of Bone Mineral, Chicago, Univ. of Chicago Press, p. 177.
Piez, K. A. (1961), *Science 134*, 841.
Porter, R. R. (1957), *Methods Enzymol. 4*, 217, 235.
Porter, R. R., and Sanger, F. (1948), *Biochem. J. 42*, 287.
Saito, S. (1960), *Bull. Tokyo Med. and Dental Univ. 7*, 385.
Sanger, F. (1945), *Biochem. J. 39*, 507.
Solomons, C., and Irving, J. (1958), *Biochem. J. 68*, 499.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Tristram, G. (1953), *Proteins 1A*, 186, 211.
Veis, A., Anesey, J., and Cohen, J. (1960), *J. Am. Leather Chemists' Assoc. 55*, 548.
Veis, A., Anesey, J., and Cohen, J. (1962), *Arch. Biochem. Biophys. 98*, 104.
Veis, A., and Cohen, J. (1956), *J. Am. Chem. Soc. 78*, 6238
Veis, A., and Drake, M. P. (1963), *J. Biol. Chem. 238*, 2003

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

ROBERT J. SCHLUETER† AND ARTHUR VEIS

From the Department of Biochemistry, Northwestern University, Chicago, Ill.

Received April 23, 1964; revised August 10, 1964

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.

* This work supported by a grant (DE-01734) from the National Institute of Dental Research, U. S. Public Health Service. This discussion is taken, in part, from a dissertation submitted by R. J. Schlueter in partial fulfillment of the requirements for the Ph.D. degree, Northwestern University, August, 1963.

† Present address: Armour Pharmaceutical Co., Kankakee, Ill.

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

TABLE I
FRAGMENTATION OF DENTINE AND CORIUM COLLAGENS BY PERIODATE OXIDATION

Collagen	Extraction Time (hr)	Fraction: Insoluble Residue PR N%[a]	Insoluble Residue PR P%[b]	Soluble, pH 2 Precipitate PS-P N%[a]	Soluble, pH 2 Precipitate PS-P P%[b]	Soluble, pH 2 Supernatant PS-S N%[a]	Soluble, pH 2 Supernatant PS-S P%[b]	Lost on Dialysis PS-D N%[a]	Lost on Dialysis PS-D P%[b]
Dentine	30	69.3	24.7	6.4	39.8	14.4	22.5	9.9	13.0
	52	17.6	6.9	4.7	41.8	24.5	38.0	52.2	13.3
Corium	30	65.5		0.4		18.4		9.1	

[a] Per cent of total nitrogen of the system. [b] Per cent of total phosphorus of the system.

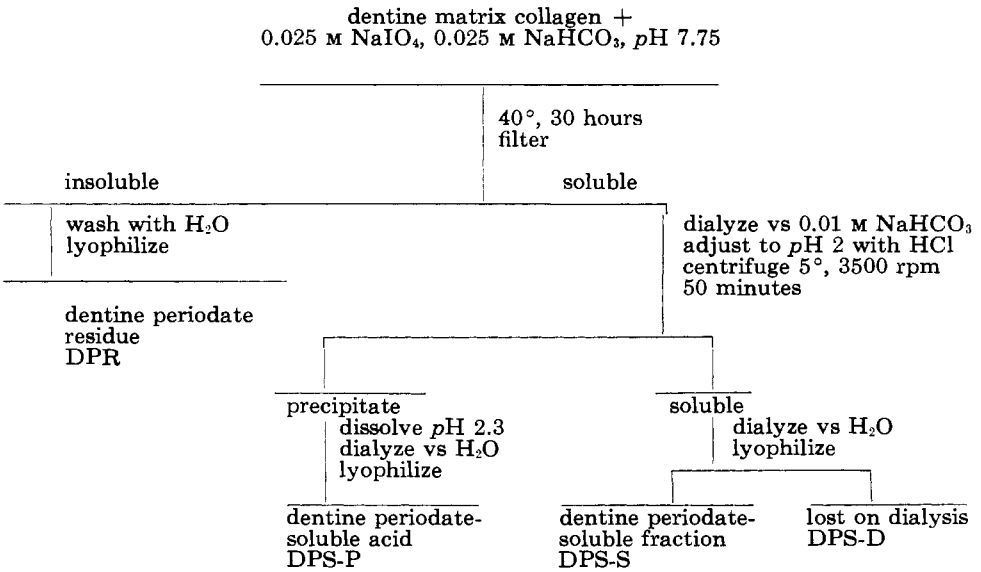

FIG. 1.—Preferred procedure for dialysis of dentine matrix collagen with sodium (meta)periodate and sodium bicarbonate. Corresponding corium fractions would be CPR, CPS, and CPS-P.

0.5 *p*H unit during the reaction. In the Grassmann-Kühn procedure the excess periodate was reduced by bubbling sulfur dioxide through the reaction mixture, with the excess sulfur dioxide being removed by subsequently bubbling nitrogen through the mixture. In addition to following this procedure, we also removed the excess periodate more simply by dialyzing the reaction mixture against 0.01 M sodium bicarbonate in the cold. The yields of the resulting fractions were the same regardless of the method used for removal of the periodate.

The sulfur dioxide treatment lowered the *p*H of the filtrate to about 1.0 and the subsequent nitrogen aeration raised the *p*H only to 1.3. This solution was dialyzed against cold distilled water for several days with repeated changes of water. Finally, the solution was dialyzed overnight against a *p*H 2.5 hydrochloric acid solution. A precipitate formed which was collected by centrifugation and lyophilized. The supernatant was also lyophilized.

In the alternate procedure, after the dialysis against 0.01 M bicarbonate, the clarified filtrate was brought to *p*H 4.5 with 6 N hydrochloric acid and then dialyzed against 15 volumes of 0.01 N (*p*H 2) hydrochloric acid. As the filtrate approached *p*H 4 it became turbid, and after further decrease in the *p*H a precipitate formed. This precipitate was collected by centrifugation (3500 rpm, 5°, 50 minutes), then dissolved in water adjusted to *p*H 5.3 with sodium hydroxide. This solution was dialyzed exhaustively against cold distilled water and lyophilized. The supernatant from the *p*H 2 precipitation was concentrated by pervaporation, dialyzed against distilled water (final *p*H, 5.3), and lyophilized.

In both procedures the residual undissolved collagen was washed with large volumes and repeated changes of distilled water, then lyophilized.

A flow diagram for the preferred dialysis procedure is given in Figure 1. In this diagram each fraction isolated is given a letter designation: R, residue; S-S, soluble supernatant; S-P, soluble precipitate; S-D, soluble, lost on dialysis. The letter prefixes in each, P or T, refer to periodate or thermal pretreatment, D and C refer to dentine or corium collagens. For example, DPS-S signifies that soluble fraction resulting from the periodate treatment of dentine, which remained soluble after the *p*H was adjusted to ≤ 2. These designations will be used throughout the following discussion.

Periodate Oxidation of Methionine.—In the course of the work some attention was focused on the possible destruction of methionine, or its reaction, during periodate treatment. A series of methionine-periodate mixtures was prepared as follows: Each system contained a solution mixture of 0.025 M sodium periodate and 0.025 M sodium bicarbonate, at *p*H 7.5, plus the following compounds in 0.125 mmole quantities: (1) methionine; (2) methionine plus glycine; (3) methionine plus glycylserine; and (4) methionine plus glycine plus glycylserine. Each mixture contained 0.0625 mmole of sodium periodate. These tubes were stoppered, covered with aluminum foil, and incubated at 40° with gentle shaking for 28.5 hours. The reaction mixtures were chromatographed by the procedure of Smith (1960). Ascending and descending techniques were employed using Whatman No. 1 paper and either phenol (453 g plus 125 ml H_2O) or butanol–acetic acid–

water (12:3:5). Spots were located with 0.5% ninhydrin in acetone and fixed with copper nitrate (Kawerau and Wieland, 1951).

Electrophoretic Mobility.—Free-moving-boundary electrophoresis was carried out in a Spinco Model H apparatus using a micro cell (2.3 ml) with a cross-section area of 0.1257 cm^2. The ionic strength was 0.1 in each case and the protein concentrations were approximately 1%. All measurements were made at 1° with a constant current of 2 ma. Mobilities were determined from the descending-limb patterns, with the boundaries measured at the position of the maximum ordinate of the gradient curves. The sign of the mobility is the same as the sign of the net charge on the protein.

Ultracentrifugal Analyses.—Sedimentation coefficients were determined in a Spinco Model E centrifuge, using a speed of 59,780 rpm. Since comparative data were sought in most cases, most runs were made with the arbitrary standard conditions of 1% protein, 0.2 ionic strength, and 40°. The elevated temperature was used to avoid problems of gelatin or aggregate formation during the sedimentation runs.

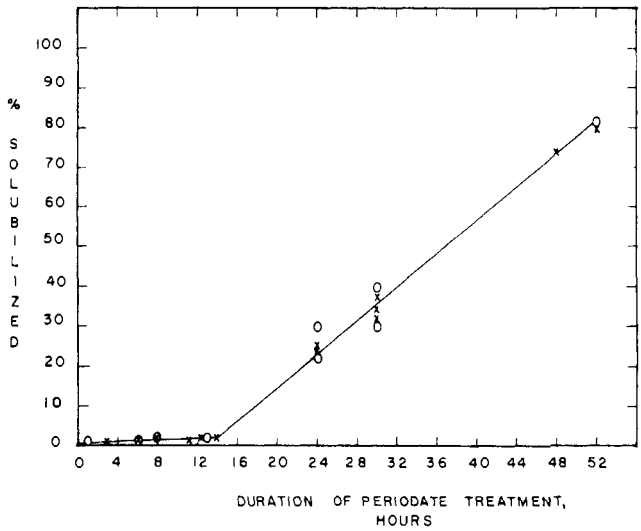

FIG. 2.—Solubilization of collagen by periodate treatment as a function of time at 40°. ×, native dentine collagen; ○, native corium collagen.

TABLE II
FRAGMENTATION OF DENTINE AND CORIUM COLLAGENS BY THERMAL HYDROLYSIS

Collagen	Extraction Time (hr, 97°)	Fraction							
		TR		TS-P		TS-S		TS-D	
		N%[a]	P%[b]	N%[a]	P%[b]	N%[a]	P%[b]	N%[a]	P%[b]
Dentine	6	45.4	27.2	40.6	31.7	6.2	3.68	10.5	37.4
Corium	2	38.4		0		53.6		7.9	

[a] Per cent of total nitrogen of the system. [b] Per cent of total phosphorus of the system.

TABLE III
COMPARISON OF PHOSPHORUS DISTRIBUTION IN THERMAL AND PERIODATE FRAGMENTATIONS OF DENTINE COLLAGEN

Treatment	Nitrogen Solubilized (%)	Per Cent Total Phosphorus in Fragment / Per Cent Total Nitrogen in Fragment (P/N)			
		R	S-P	S-S	S-D
Thermal, 97°, 6 Hours	56.6	0.60	0.78	0.59	3.56
Periodate, 30 Hours	30.7	0.35	6.22	11.56	1.21
52 Hours	82.4	0.39	8.89	1.55	0.25

Schiff's Reagent and Spot Test.—Schiff's reagent was prepared by the method of Lillie (1951). One g of basic fuchsin and 1.9 g of sodium metabisulfite were added to 100 ml of 0.15 N hydrochloric acid and shaken for 2 hours. Activated charcoal (500 mg) was then added and the mixture was shaken for an additional 2 minutes. The suspension was filtered, giving a water-clear filtrate of the reagent. This solution is reported to be stable for several months if stored at 0-5°.

The McManus and Hoch-Ligeti (1952) spot test for aldehydes, consisting of adding 5 parts of Schiff's reagent to 1 part of an aqueous test solution or suspension, was used. A stable violet or reddish-violet color was considered a positive test for the presence of aldehyde.

RESULTS

Solubilization and Fractionation.—The treatment of both intact corium and dentine matrix collagens with periodate, under the reaction conditions described, led to the partial solubilization of both collagens. There was a distinct induction period of about 14 hours (Fig. 2) after which the amount solubilized increased linearly. Within the experimental precision there appeared to be no difference in the rate of solubilization of the two types of collagen, although, as indicated in the first paper (Veis and Schlueter, 1964), the corium collagen was much more susceptible to thermal hydrolysis than was the dentine collagen. In the 52-hour periodate treatment at 40°, neither the corium collagen nor the dentine collagen showed any solubility in buffer system minus the periodate reagent.

The products of periodate treatment were processed according to the procedure outlined in Figure 1. Each fraction was monitored for total nitrogen and phosphorus content. The results of these analyses are indicated in Table I in terms of the percentage of the total system nitrogen or phosphorus found in each fraction. In the case of the corium collagen, there was, of course, no phosphorus to measure. It is of special interest to note that the corium yielded only a very small CPS-P fraction, although the total nitrogen solubilized in the CPS-S and CPS-D fractions was slightly greater than the total nitrogen of all soluble fragments from the dentine collagen. The phosphorus was concentrated largely in the DPS-P fraction, particularly in the early stages of the periodate treatment. As degradation proceeded, more of the phosphorus appeared in the DPS-S fraction. There was no

TABLE IV
CHANGES IN AMINO ACID COMPOSITION OF DENTINE COLLAGEN FOLLOWING PERIODATE OXIDATION

Amino Acid	Amino Acid Content (g residue/100 g protein)				
	Native Collagen		Insoluble Residue	Soluble Undialyzed Corrected	Calculated Composition of Periodate-treated Protein[a]
Lysine	2.83		2.72	2.52	2.65
Hydroxylysine	1.56		0.23	0.15	0.21
Histidine	0.84		0.79	0.70	0.76
Arginine	8.57		7.87	6.61	7.47
Aspartic acid	6.73		6.14	8.10	6.73
Threonine	2.01		1.70	1.28	1.57
Serine	3.89	3.98	3.07	2.71	2.96 3.54
Phosphoserine	0.09		0.08	1.74	0.58
Glutamic acid	10.72		10.68	9.44	10.29
Proline	13.41		11.65	10.46	11.28
Hydroxyproline	13.09		10.91	7.29	9.79
Glycine	21.82		20.88	20.96	20.89
Alanine	10.35		9.08	10.33	9.46
Valine	2.41		2.13	2.14	2.13
Methionine	0.59	(0.96)[b]	0.56	0.17	0.44
Isoleucine	1.48		1.24	1.23	1.23
Leucine	3.28		2.92	2.67	2.83
Tyrosine	0.76		0	0	0
Phenylalanine	2.05		1.71	1.73	1.71
Sarcosine			0.14	0.22	0.15
α-NH_2-*n*-butyric acid				0.12	0.04
Cystathionine			0.42	0.44	0.42
Ammonia	0.83		0.96	2.23	1.34
Phosphate (HPO_3)	1.08		0.37	2.97	1.17
Methionine sulfoxides	0.37				

[a] Based on 69.3% insoluble, data of Table I. [b] Methionine plus methionine sulfoxides.

appreciable increase in the dialyzable phosphorus (DPS-D), although after 52 hours 30% of the collagen had been degraded to small dialyzable peptides.

When the DPS-P fraction was found, the question arose as to the possible appearance of such a fraction from a thermal hydrolysis. Conditions were chosen such that about 40% of both corium and dentine matrix collagen would be solubilized. This required extraction at 97° for 6 hours in the case of the dentine collagen and for 2 hours in the case of the corium collagen. Appropriate dialysis and *p*H adjustment, as outlined in Figure 1, did lead to the appearance of a precipitate fraction, DTS-P. The nitrogen and phosphorus distribution data from the thermal degradations are given in Table II.

A comparison of the per cent phosphorus to per cent nitrogen ratio for each fraction in each treatment, Table III, shows both an accumulation of phosphorus containing moieties in the DS-P and DS-S fractions and the distinctly different modes of structural degradation or depolymerization by periodate oxidation and thermal hydrolysis. The high-molecular-weight (non-dialyzable) thermal fractions all have nearly constant P/N ratios. In contrast to the periodate oxidation, thermal hydrolysis releases a large fraction (37%) of the phosphate in dialyzable form.

Composition of the Fractions.—A number of complete amino acid analyses were carried out to determine two points in particular: the effect of the periodate treatment on the individual amino acids, and the differences in composition of the various fractions. The amino acid–destruction question is answered by the data of Table IV in which the compositions of the periodate-treated fractions are compared with that of the native dentine collagen. The composition of the "solubilized dentine" represents the combined DPS-P, DPS-S, and DPS-D fractions, having been determined on an aliquot of the soluble material before dialysis. The values in the table have been corrected for the salt content of the undialyzed fraction. In agreement with the work of Hörmann and Fries (1958), the hydroxylysine content of the periodate-treated collagen was decreased to 13% of its original value. In addition, and in marked disagreement with the report of Grassmann and Kühn (1955), tyrosine was found to be completely destroyed. Hörmann *et al.* (1959) have shown that periodate treatment of calfskin procollagen in an acid medium leads to the slow destruction of its hydroxylysine, tyrosine, and methionine. Table IV further shows that hydroxyproline, proline, histidine, threonine, methionine, leucine, and phenylalanine were all reduced significantly. New chromatographic peaks appeared corresponding to sarcosine, cystathionine, and α-NH_2-*n*-butyric acid. Additional ammonia was noted. There also appeared to be a small loss in serine but this was difficult to verify because of the appearance of more phosphoserine in the hydrolysate of the soluble fraction. In line with the observation that solubilization of the dentine and corium collagens by periodate treatment was similar, the composition of the corium collagen was affected in the same fashion. In particular, tyrosine was totally destroyed and hydroxylysine was markedly reduced. There were small decreases in arginine, histidine, methionine, phenylalanine, and hydroxyproline. Again, sarcosine, α-NH_2-*n*-butyric acid, and cystathionine were present following periodate treatment. There was no "phosphoserine" appearing in the soluble periodate-treated corium, demonstrating that the "phosphoserine" peak present in the chromatograms of periodate-treated dentine was not an artifact.

The compositions of the specific fractions following periodate treatment, in residues per 1000 residues, are given in Table V. In assessing these data it should be kept in mind that the dialyzable peptides of low molecular weight, containing 8–30% of the total nitrogen of the various systems (Table I), are not represented. All of the essential features of the fraction compositions are illustrated by the data referring to the 30-

TABLE V
COMPOSITION OF FRAGMENTS RESULTING FROM PERIODATE OXIDATION

	Amino Acid Content (residues/1000 residues)										
	Dentine							Corium			
		30-Hour Treatment			52-Hour Treatment				30-Hour Treatment		
	Native	PR	PS-P	PS-S	PR	PS-P	PS-S	Native	PR	PS-P	PS-S
Lysine	18.9	19.4	24.4	20.0	18.4	21.1	16.1	24.1	23.2	24.6	21.6
Hydroxylysine	9.2	1.4	0.9	2.2	1.8	2.8	1.3	5.8	1.2	1.0	1.1
Histidine	5.2	5.3	6.2	3.7	6.4	3.8	3.7	5.8	6.0	3.7	0.9
Arginine	46.9	46.0	44.2	33.0	45.5	46.3	37.3	48.7	46.3	41.9	38.7
Aspartic acid	49.9	48.6	80.4	78.9	53.4	77.3	55.1	45.5	48.9	62.0	54.6
Threonine	17.0	15.4	15.8	15.8	16.9	16.4	16.7	16.1	15.1	19.1	16.9
Serine	38.2	32.2	59.4	51.4	34.1	56.6	32.9	34.8	30.6	34.9	34.9
Phosphoserine	0.5	0.5	4.3	2.8	0.9	7.8	0.6	T	T	T[a]	T
Glutamic acid	70.9	75.4	83.5	81.2	82.6	85.1	90.8	75.3	76.5	88.0	85.9
Proline	117.8	109.4	90.5	100.1	118.5	108.3	109.1	123.0	127.7	117.4	111.1
Hydroxyproline	98.7	88.0	62.8	80.8	83.9	84.7	83.9	88.7	79.7	64.6	92.1
Glycine	325.8	333.3	327.7	335.7	342.3	308.2	353.9	343.4	346.8	319.9	347.4
Alanine	124.2	116.4	118.8	120.8	116.4	102.1	128.8	115.5	126.7	115.2	124.3
Valine	20.7	19.6	25.1	23.4	20.2	18.9	19.8	19.0	20.0	26.2	20.8
Methionine	5.9[b]	3.9	2.8	2.6	3.3	2.4	2.0	5.4[b]	3.2	3.3	3.3
Isoleucine	11.1	10.0	8.9	10.5	9.6	8.6	8.7	11.4	10.4	15.4	11.0
Leucine	24.7	23.6	23.4	22.3	26.7	29.1	24.4	23.5	23.7	36.7	22.3
Tyrosine	4.0	0	0	0	0	0	0	4.0	0	0	0
Phenylalanine	11.9	10.6	11.2	9.9	13.2	13.3	10.8	11.9	10.6	15.2	10.1
Sarcosine		1.8	10.0	4.4	2.1	4.1	T		1.3	5.8	T
α-NH_2-*n*-butyric acid			1.1	1.0	0.7	1.3	1.1			1.8	2.0
Cystathionine		2.1	3.0	2.6	3.0	2.1	3.0		2.0	3.3	1.2
Ammonia[c]	(45.8)	(54.7)	(70.7)	(73.7)	(59.0)	(88.7)	(62.9)	(41.9)	(51.2)	(75.4)	(58.5)
Phosphate[c]	(5.8)	(4.4)	(41.5)	(36.0)	(5.0)	(38.0)	(4.3)	(T)	T	T	T

[a] These trace amounts may be artifact. [b] Includes methionine sulfoxide. [c] Equivalents of HPO_3 per 1000 amino acid residues.

hour dentine periodate treatment. In this case the DPS-P and DPS-S fractions account for 21% of the total nitrogen of the system, the residue (DPR) represents 69%, and the remainder was lost on dialysis. The most remarkable aspects of these data were the marked increases in the aspartic acid, serine, and glutamic acid contents of the DPS-P and DPS-S fractions, as well as the accumulation of phosphate in these soluble fractions. Of the new constituents, sarcosine was concentrated in the soluble fractions, whereas the cystathionine was more uniformly distributed. Since methionine sulfoxides were never found in the periodate-treated collagens, it seemed likely that the cystathionine, which appeared in an amount equivalent to the methionine decrease, was formed from the methionine. Conceivably, the methionine could have been split to a compound such as homocysteine which then reacted with a seryl residue to form the cystathionine. The decrease in combined serine and phosphoserine contents of 0.44% (Table IV) is also of the proper magnitude for pleasing stoichiometry. However, direct methionine oxidation studies, with methionine alone or with glycylserine or glycine, did not confirm this explanation. Chromatography of the reaction mixtures exhibited no components in addition to the starting materials and methionine sulfoxide. The origin of the sarcosine is also unaccounted for.

As the periodate reaction period was extended to 52 hours, relatively larger amounts of the insoluble dentine collagen were, in effect, transferred to the PS-S and PS-D fractions. Since there was relatively little net destruction of amino acids the compositions of these fractions tend to approach the composition of the residual and the untreated collagen.

The Fate of the Carbohydrate.—After periodate treatment the dentine collagen was subjected to analysis for residual hexose content with anthrone. A definite colored anthrone complex was developed, but the reaction mixtures were an intense orange rather than the usual green color. Using acid-soluble collagen, Hörmann and Fries (1958) found periodate treatment to lead to rapid destruction of the hexoses. Over 75% of the hexoses were destroyed in the first 0.5 hour of the reaction period. The hexose content then remained stable over the next 35-hour period. Analyses during this time were entirely normal, the anthrone-hexose mixture giving the typical absorption spectrum with a maximum at 625 mμ. In the present experiments with dentine collagen, the absorption spectrum of the periodate-treated collagen was entirely different from that of the untreated collagen, with an absorption maximum at 540 mμ rather than 625 mμ. The spectra of the anthrone reagent-hexose mixtures are illustrated in Figure 3, where the spectra of glucose, untreated dentine collagen, periodate-treated collagen, and anthrone containing some sodium periodate are compared. Small quantities of periodate do react in the anthrone-hexose assay to give a characteristic absorption spectrum with a maximum of 587 mμ and a 540-mμ shoulder. Upon standing, this mixture turns brownish-black and a black precipitate settles out. No such precipitates were noted in the periodate-treated collagen analysis. Prolonged dialyses assured the removal of all the periodate from the DPS-S fraction whose anthrone spectrum is shown in Figure 3. Helbert and Brown (1956, 1957) reported hexuronic acids to have the spectrum shown for the DPS-S fraction in the anthrone-reagent mixture. It thus seems likely that some of the hexoses in the dentine collagen are protected in such a way (by 1–3 or 2–4 bonding) that the pyranose ring is not oxidized, while oxidiation takes place at the C_6 position.

After isolation the DPS-P and DPS-S fractions gave a negative Schiff test, showing the absence of aldehydes. However, the DPR and CPR, as well as native

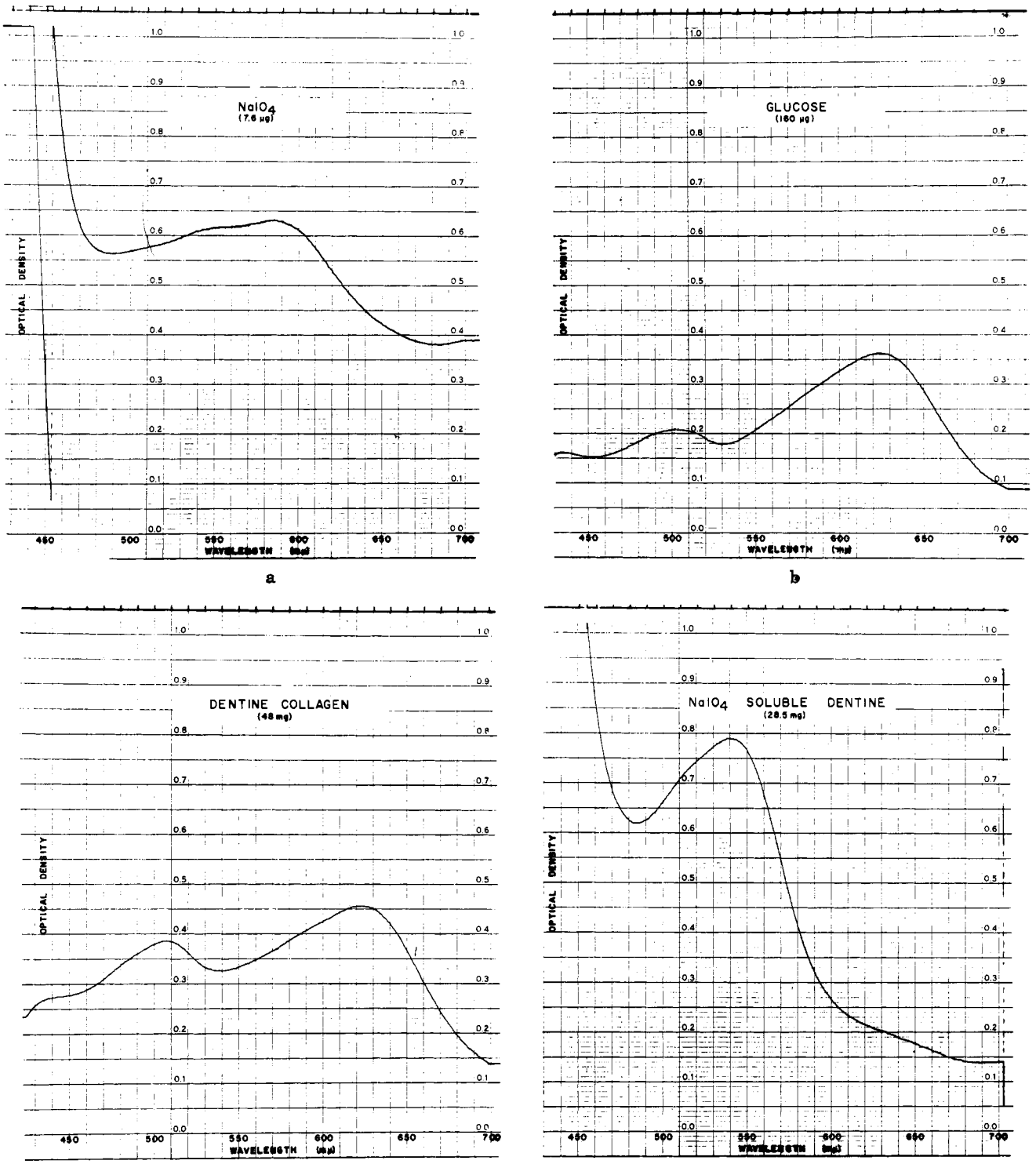

FIG. 3.—Spectra of anthrone reagent–collagen mixtures before and after periodate treatment.

samples of both dentine and corium collagens, gave positive results in the Schiff test. The color developed by the intact collagens was less intense than that of the periodate-treated collagens and tended to fade more rapidly.

Characterization of the Periodate Fragments.—Since there seemed to be a preferential release of phosphate-containing moieties into the soluble fractions, and since we had speculated that the phosphate groups were part of the cross-linking mechanism in the intact dentine, it was of interest to use the same solubilization and swelling tests on the periodate residues as had been used on the native collagen. At 60° where, as shown in Figure 4, native dentine collagen is essentially insoluble in water, the insoluble DPR fraction is more soluble than is native corium collagen under the same conditions (Veis and Cohen, 1955). There was no evidence for an induction period during which hydrolysis took place without concomitant solubilization. Such an induction period had been noted in 80° hydrolyses of untreated dentine collagen (paper I). These data suggest that extensive cross-linkage or main-chain cleavage had resulted from the oxidative treatment.

Swelling studies, depicted in Figure 5, did not appear to agree with this suggestion. There was no increase in the acid swelling of the DPR, and the CPR swelling

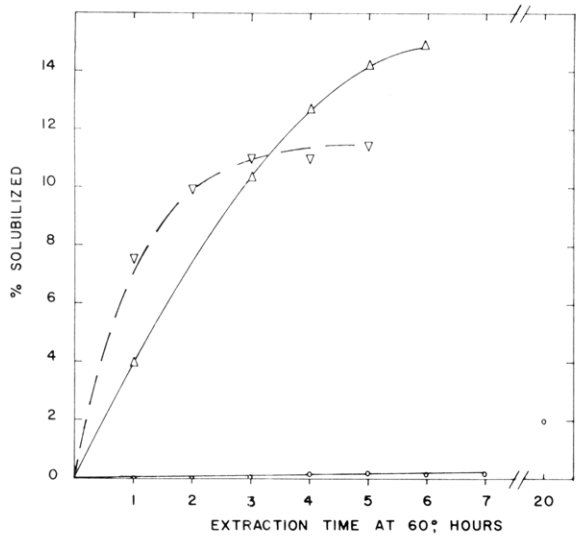

FIG. 4.—Solubilization of dentine collagen at 60° following periodate treatment in comparison with the solubilization of native dentine and native corium collagens. O, native dentine collagen; ▽, native corium collagen (data of Veis and Cohen, 1955); △, periodate-treated dentine collagen, 30-hour residue (DPR).

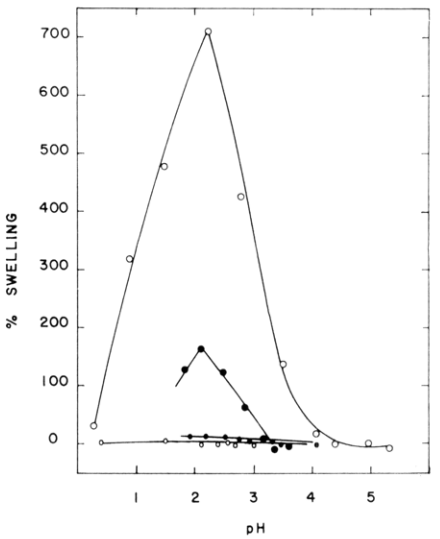

FIG. 5.—Acid-swelling behavior of periodate-treated insoluble residues. •, DPR; ●, CPR; ○, native dentine collagen; O, native corium collagen.

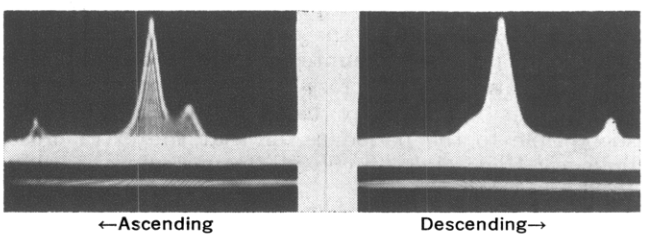

FIG. 6.—Electrophoretic analyses of periodate-solubilized dentine collagen. Photographs taken after 120 minutes. *p*H 5.3 acetate buffer system, 0.1 ionic strength.

was markedly reduced in comparison with the native corium collagen. These data, together with the solubilization information, indicate that while the very strong cross-linking regions in the native structures are disrupted by the periodate oxidation, a secondary cross-linking with weaker, more readily hydrolyzable bonds occurs during the oxidative degradation. The most likely source of the cross-linking reagent is the formaldehyde produced during the degradation of both the hexoses and the hydroxylysine (Hörmann and Fries, 1958). Veis and Drake (1963) have shown that monofunctional aldehydes can readily cross-link collagen both intra- and intermolecularly.

Upon electrophoresis at *p*H 5.3 in 0.1 M sodium acetate buffer, two components were evident in the 30-hour-total periodate-solubilized material (after dialysis but before separation into DPS-P and DPS-S fractions). Both components illustrated in Figure 6 were anionic, the smaller fast component had a mobility of -14.0×10^{-5} cm²/v-sec, the slower component a mobility of -4.3×10^{-5} cm²/v-sec. The phosphate-free periodate-solubilized corium collagen under the same conditions showed only a single component with a mobility of -2.34×10^{-5} cm²/v-sec. The DPS-S fraction showed only a trace of the fast component and was principally the -4.1×10^{-5} cm²/v-sec component. We have not been able to obtain reasonable electrophoretic runs of the DPS-P fraction since it tends to gel under the operating conditions used. Both fast and slow components were clearly evident, however.

Sedimentation coefficients were determined for the 30-hour and 52-hour DPS-P fractions at 1% concentration in 0.05 M $NaHCO_3$, 0.1 M KCl systems at 40°. The two components were not resolved by this method; a single broad peak was observed in each case. There was a marked difference in the sedimentation rates, $s_{30\,hour} = 1.96$ S and $s_{52\,hour} = 3.28$ S.

Discussion and Conclusions

The working hypothesis for this and most other current work on the native collagens is based on a model of the fibril structure in which the long, asymmetric monomer collagen rods, the tropocollagen units, are packed into a very particularly ordered array. The maturation process is thought to consist of the imposition of both intramonomer and intermonomer covalent cross-linkages within this ordered array. The intermonomer cross-linkages convert the fibril from an acid-soluble system to an isoluble three-dimensional infinite network. The comparisons of swelling and solubility of dentine and corium collagens reported in paper I indicated that the dentine collagen systems were more highly cross-linked than the corium collagens. It was suggested that the set of cross-linkages present in the corium was present in the dentine collagen, but that an additional set of bonds of different chemical character increased the degree of polymerization of the dentine matrix. From this point of view the solubilization data of Figure 2, showing both corium and dentine collagen to be solubilized by periodate oxidation at the same rate and with an equivalently long induction period, are not so surprising, although more than one explanation is tenable at this point.

First, let us suppose that the cross-linkages common to the corium and dentine collagens are formed via the hexoses, as suggested by Grassmann and Kühn (1955) in such a way that these may be oxidized by periodate (1–2, 1–4, or 1–6 bonding). The rate of degradation of such hexoses should then be expected to parallel the rate of breakdown of the hexoses in the nonbonded soluble collagens. As shown by Hörmann and Fries (1958), Blumenfeld *et al.* (1963), and Bose (1963), this reaction can be accomplished within a few hours. Grassmann and Kühn (1955) also showed the hexoses of fibrous corium collagen to be oxidized within 1.5 hours under conditions similar to those used here. Solubilization should not have shown a 14-hour induction period in this case for either collagen. Further,

if the extra stabilizing bonds in the dentine matrix were of different chemical character, not subject to periodate oxidation, then solubilization of the dentine should have been delayed.

An alternative situation is to suppose that the common set of cross-linkages does not involve periodate-susceptible hexoses, then solubilization might proceed by the oxidative degradation of the polypeptide backbone, either in the region of the cross-linkages or between branch points. Strong support for this supposition comes from the work of Grassmann and Kühn (1955), who reported very low molecular weights for the solubilized collagen; from the work of Blumenfeld *et al.* (1963), who showed that in ichthyocol, at least, the hexoses were not protected from periodate oxidation; and from the present study in which clear-cut evidence is given for the destruction of some amino acids. These amino acid–degradation reactions are considerably slower than the hexose oxidation. For example, only one-half of the hydroxylysine of procollagen is oxidized within 5 hours and the reaction slows so that 80–90% destruction requires more than 50 hours (Hörmann and Fries, 1958). In contrast, the aqueous periodic acid–Schiff test stains native collagen to its maximal level within 5–10 minutes. The equa rate of solubilization of the dentine and corium collagens could then be accounted for either by supposing that the extra dentine cross-links were located within the regions bounded by backbone bonds especially susceptible to the periodate, or that the extra cross-linkages themselves involved groups more rapidly oxidized by the periodate.

A second basic element of our working hypothesis is that the covalently bound phosphorus is involved in the cross-linking mechanism (Veis and Schlueter, 1964). Enzymatic studies supporting this position are to be described in paper III of this series. Therefore the phosphorus distribution data should serve as a marker for the distribution of chain segments which contain, or contained, the cross-linkages prior to oxidative degradation. While, as shown in Table III, there was a relative enrichment of phosphorus in the dialyzable peptide fraction, evidence is clear-cut for a marked accumulation of the phosphorus in the DPS-P fraction, the smallest fraction in terms of nitrogen content. The DPS-P fraction is also particularly rich in serine, aspartic acid, and glutamic acid. The DPS-P, on the other hand, showed no increase in threonine content, and a depletion of the hydroxyproline. The presence of small amounts of a component with a mobility of $\sim -14 \times 10^{-5}$ cm^2/v-sec suggests that the phosphate groups may be even more specifically confined in a highly localized fashion to a few chain segments. The DPS fractions also contain the partially protected pyranose-ring structures and suggest a phosphate-hexose bond. It is not worthwhile to consider the composition question further at present because it is evident from the swelling data that the aldehydes produced during the oxidation of both hexoses and hydroxylysine can add new cross-linkages to the system. If the aldehyde cross-linkages are formed simultaneously with structure degradation, then the isolated peptides may have a direct relationship with the situation prevailing in the intact tissue. However, subsequent aldehyde cross-linking could occur after structure randomization. This problem remains for further study.

The site of periodate attack on the peptide backbone appears to involve regions rich in arginine and histidine. Comparison of the compositions of the total 30-hour-soluble dentine fractions with the DPS-P and DPS-S fraction compositions show that the dialyzable peptides (DPS-D) are particularly enriched in arginine and histidine, and this is in the face of the fact that the overall composition data indicate some destruction of both these amino acids by 10–12%. It is also noteworthy that tyrosine is completely destroyed. Schmitt and his colleagues (Hodge *et al.*, 1960; Rubin *et al.*, 1963) and Nishihara and Miyata (1962) have proposed that the tyrosine of collagen is localized in atypical (low pyrrolidine-ring content) peptide sequences at the ends of the monomers and that these sequences control the aggregation of the monomer units. The end-regions of the collagen monomers accept phosphotungstic acid and similar stains quite avidly, showing the basic nature of these regions (Hodge and Schmitt, 1960; Bensusan *et al.*, 1962). These observations are compatible with the present data, which point to the extensive degradation of tyrosyl residues and the formation of basic peptides of low molecular weight as leading to solubilization of the collagen matrix in both corium and dentine. Thus the set of cross-linkages common to both collagens may involve the end-regions, whereas the phosphorylated regions and "extra" dentine cross-linkage sites may be distributed in localized segments within the organized body of the monomer units, sharply limiting the swelling ability of both native and DPR-insoluble dentine.

Random thermal hydrolytic scission of the dentine-collagen backbone provides fractions with quite uniform phosphate-content distributions, except for the DTS-D fraction. The high content of phosphate in that fraction indicates that about 40% of the phosphate is easily hydrolyzed and may not be involved in diester cross-link formation. The even distribution of the P/N ratios in the remaining soluble high-molecular-weight and insoluble fractions emphasizes the specificity of the periodate attack on the peptide backbone. The ease of solubilization of the DPR-insoluble fraction upon subsequent mild thermal hydrolysis shows that the peptide backbone of the cross-linked network has been seriously degraded by the periodate treatment and accounts for the fact that Grassmann and Kühn (1955) found the insoluble residues from their periodate treatment of corium collagen to have an average intact-chain length of only 25 residues. Such a low chain length is compatible with the insolubility of the residue only if one assumes a very high cross-link density.

The arguments presented above lead us to the following tentative conclusions. The corium and dentine collagens must have a common set of intermolecular cross-linkages in similar number and distribution. These cross-linkages exist in regions susceptible to periodate oxidation, possibly in the tyrosine-rich so-called "end-chain" or "telopeptide" loci. Periodate does not solubilize collagen by the oxidation of the hexoses; oxidative degradation occurs within the peptide chains and particularly at tyrosyl residues. Phosphate moieties occur at sites in between periodate-susceptible regions. These phosphate esters, probably attached to serine, occur in groups with a high charge density. Somewhat more than half the phosphate esters are resistant to thermal hydrolysis and may thus be involved in diester cross-linking. Some hexose, also present in the phosphate-containing peptide chains, appears to be resistant to periodate oxidation and may also be involved in the extra or additional set of cross-linkages in the dentine-collagen matrix.

References

Bensusan, H. B., Mumaw, V. R., and Scanu, A. W. (1962), *Biochemistry 1*, 215.

Blumenfeld, O. O., Paz, M., Gallop, P. M., and Seifter, S. (1963), *J. Biol. Chem. 238*, 3835.

Bose, S. M. (1963), *Biochim. Biophys. Acta 74*, 265.
Grassmann, W., and Kühn, K. (1955), *Z. Physiol. Chem. 301*, 1.
Helbert, J., and Brown, K. (1956), *Anal. Chem. 28*, 1098.
Helbert, J., and Brown, K. (1957), *Anal. Chem. 29*, 1464.
Hodge, A. J., Highberger, J. H., Deffner, G. G. J., and Schmitt, F. O. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 197.
Hodge, A. J., and Schmitt, F. O. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 186.
Hörmann, H., and Fries, G. (1958), *Z. Physiol. Chem. 311*, 19.
Hörmann, H., Hannig, K., and Fries, G. (1959), *Z. Physiol. Chem. 315*, 109.
Kawerau, E., and Wieland, T. (1951), *Nature 168*, 77.
Lillie, R. (1951), *Stain Technol. 26*, 163.
McManus, J., and Hoch-Ligeti, C. (1952), *Lab. Invest. 1*, 19.
Nishihara, T., and Miyata, T. (1962), *Collagen Symp.* 3, 66.
Rubin, A. L., Pfahl, D., Speakman, P. T., Davison, P. F., and Schmitt, F. O. (1963), *Science 139*, 37.
Smith, I. (1960), Chromatographic and Electrophoretic Techniques, Vol. I, London, Heinemann, p. 20.
Veis, A., and Cohen, J. (1955), *J. Am. Chem. Soc. 77*, 2364.
Veis, A., and Drake, M. P. (1963), *J. Biol. Chem. 238*, 2003.
Veis, A., and Schlueter, R. (1964), *Biochemistry 3*, 1650 (this issue; preceding paper).

Optical Rotatory Dispersion Studies of Poly-L-tyrosine and Copolymers of L-Glutamic Acid and L-Tyrosine. Significance of the Tyrosyl Cotton Effects with Respect to Protein Conformation*

GERALD D. FASMAN,† ERIKA BODENHEIMER, AND CAROLE LINDBLOW

From the Graduate Department of Biochemistry,‡ Brandeis University, Waltham, Mass.

Received April 1, 1964; revised August 14, 1964

The synthesis of high-molecular-weight (60,000–125,000) poly-L-tyrosine and copolymers of L-tyrosine and L-glutamic acid (random and block) is reported. Poly-L-tyrosine can exist in either a helical or a random-chain conformation in the un-ionized form. The optical rotatory dispersion (ORD) of helical poly-L-tyrosine (in 0.2 M NaCl, *p*H 11.2) over the wavelength range 500–227 mμ is recorded. Multiple Cotton effects are observed with peaks at 286 mμ, $[m']_{286}$ = 2650°, and 254 mμ, $[m']_{254}$ = 4240°, and a trough at 238 mμ, $[m']_{238}$ = −6410°. Upon ionization of the phenolic hydroxyls of poly-L-tyrosine the positive peak of the Cotton effect at 286 mμ vanishes, the peak at 254 mμ diminishes, and simultaneously the trough at 238 mμ decreases. This probably represents a helix → random-coil transition. The b_o changes from +570 to +413 during this structural change. Ultraviolet-absorption spectra of the helical polypeptide indicate that the pK_a of the tyrosyl hydroxyl is much higher than that of the monomer, and a red shift of 2 mμ (λ_{max} = 277 mμ) is observed. The ORD and ultraviolet spectra suggest that tyrosyl-tyrosyl interactions occur in the helical conformation. ORD and ultraviolet spectra are also reported for films of poly-L-tyrosine. The negative Cotton effect (trough, 238 mμ) further suggests that poly-L-tyrosine is a right-handed helix. The tyrosyl Cotton effect (peak, 286 mμ) is not observed in copolymers of L-tyrosine and L-glutamic acid until 20 mole % tyrosine is present. Although this Cotton effect is therefore unlikely to be observed in proteins, the b_0 and $[m']_{233}$ values can be significantly altered and the interpretation of such values, for proteins with high tyrosine contents, may be complex.

Optical rotatory dispersion (ORD)[1] measurements have become extensively used to determine the conformation of proteins (see Urnes and Doty, 1961; Blout, 1960; Fasman, 1963). The anomalous rotatory dispersion curves for many synthetic poly-α-amino acids and proteins in the helical conformation have been adequately interpreted by the equation proposed by Moffitt (Moffitt, 1956; Moffitt and Yang, 1956), where mrw = mean residue weight.

$$[m'] = \frac{\text{mrw}}{100} \cdot \frac{3}{n^2 + 2} \cdot [\alpha] = a_o \frac{\lambda_o^2}{\lambda^2 - \lambda_o^2} + b_o \left[\frac{\lambda_o^2}{\lambda^2 - \lambda_o^2}\right]^2$$

* This work was supported in part by grants from the National Science Foundation (GB-428), the U. S. Public Health Service Research Grant from the National Institute of Arthritis and Metabolic Diseases (AM-05852), and the American Heart Association (63G89).

† This work was done during the tenure of an Established Investigatorship of the American Heart Association.

‡ Contribution No. 315.

[1] Abbreviation used in this work: ORD, optical rotatory dispersion.

The constant, b_o, in this equation has been used as a measure of the helical content in polypeptides and proteins (Cohen and Szent-Gyorgyi, 1957; Blout and Karlson, 1958; Doty, 1960). The sign of b_o has been taken to signify the sense of helix. The value for b_o of approximately −630 has been found for the right-handed helical conformation of many synthetic poly-α-amino acids (Moffitt, 1956; Moffitt and Yang, 1956; also see Urnes and Doty, 1961) and several proteins, assumed to be completely helical (e.g., tropomyosin) (Cohen and Szent-Gyorgyi, 1957; Kay and Bailey, 1959).

Positive b_o values have been reported for several poly-α-amino acids; poly-L-tyrosine (Elliott *et al.*, 1957; Downie *et al.*, 1959; Coombes *et al.*, 1960; Katchalski, 1959; Fasman, 1962), poly-β-benzyl-L-aspartate (Blout and Karlson, 1958; Karlson *et al.*, 1960; Bradbury *et al.*, 1960), poly-*N*-benzyl-L-histidine (Norland *et al.*, 1963) and poly-L-tryptophan (Sela *et al.*, 1961). Left-handed helices of either L- or D-amino acids would be expected to have positive b_o values. Poly-β-benzyl-L-aspartate (b_o = +630) was shown to exist as a left-handed helix (Karlson *et al.*,