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Peptides Isolated from a Partial Hydrolysate of Steer Hide Collagen. II. Evidence for the Prolyl-Hydroxyproline Linkage in Collagen

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The existence of a linkage between residues of proline and hydroxyproline in collagen has been established by the isolation and identification of gly-pro-hypro and gly-(hypro,pro)-gly as the DNP derivatives from a partial acid hydrolysate of steer hide collagen. As a result of these findings, the sequence -gly-pro-hypro- should be considered in any proposed structure of collagen. Twenty-eight peptides are reported for the first time and only eleven peptides of a total of 48 (including 17 tripeptides and one tetrapeptide) agree with the sequence –P–G–R–. The data lend further support to a recent report that the repetitive sequence –P–G–R– is not an essential requirement for the structure of collagen.

Schroeder, Kay, LeGette, Honnen and Green[1] have presented evidence that the sequence -gly-pro-hypro-gly-[2] may be of frequent occurrence in collagen and gelatin. We wish to report the identification of the tripeptide, gly-pro-hypro, and the isolation of the tetrapeptide, gly-(hypro,pro)-gly, from a partial hydrolysate of steer hide collagen. All the peptides were isolated as the dinitrophenyl (DNP) derivatives and quantitative information has been obtained in some instances.

Experimental

Fractionation of Partial Acid Hydrolysate.—The collagen, purified as described previously,[3] was hydrolyzed with concentrated hydrochloric acid for four days at 37°. After removal of the excess acid, the hydrolysate was separated into four fractions—aromatic, basic, acidic and neutral—by passage of the hydrolysate successively through columns of activated charcoal, a cation-exchange resin and an anion-exchange resin. The activated charcoal was equilibrated with 5% acetic acid; the cation exchanger (XE-64, Rohm and Haas Company) was buffered at pH 6.0 with 0.2 N ammonium acetate and rinsed free of excess buffer; the anion exchanger (IR-4B, Rohm and Haas Company) was pretreated with 10% acetic acid and washed with water until the effluent pH reached 3.5. The aromatic fraction was eluted with 5% phenol in 20% acetic acid, the basic fraction with N ammonium hydroxide, and the acid fraction with 0.6 N hydrochloric acid. The neutral fraction consisted of the unadsorbed material. The percentage distribution of the nitrogen in the four fractions was: aromatic 0.8; basic 21.8; acid 8.2 and neutral 67.5. The nitrogen content of the neutral fraction appears to be high because of incomplete adsorption of the acidic fraction on the IR-4B column. Acidic peptides have been found in the neutral fraction and are reported below.

The acid fraction was resolved into ten sub-fractions by chromatography on Dowex-50 using the method of Moore and Stein[4] except that the column was run at room temperature.

All the fractions were dinitrophenylated by the method of Sanger.[5]

Chromatography of DNP Derivatives.—The DNP derivatives of the four largest sub-fractions of the acid fraction were run on columns of buffered Super-cel[6] using either ethyl acetate saturated with water or the methyl ethyl ketone system of Mills[7] as the eluents. When the column separation was imperfect and mixed bands resulted, resolution was finally achieved by paper chromatography. Unbuffered Whatman No. 1 filter paper was used with the solvent system benzene–acetic acid–water (1:1:1)[8] and the same type of paper buffered at pH 4 with 0.2 M acetate buffer was used with t-amyl alcohol saturated with the same buffer as the developer. The latter system has been most useful for separation of slowly moving DNP-peptides and for the identification of DNP-amino acids.

The basic fraction yielded ether-soluble and acid-soluble DNP derivatives. The acid-soluble fraction should contain DNP-arginine, DNP-histidine and peptides of these basic amino acids in which the arginine or the histidine may be the N-terminal amino acid or be in the peptide chain. This fraction was resolved on Super-cel-pH 7.0 columns with methyl ethyl ketone–chloroform as the eluent.[7] The ether-soluble fraction should contain α, ϵ-di-DNP-lysine, α, ϵ-di-DNP-lysyl peptides and peptides containing ϵ-DNP-lysine with an ether-soluble DNP-amino acid as the N-terminal amino acid. Inconclusive data were obtained from this fraction and are not reported.

The neutral fraction was also divided on the basis of ether and acid solubility. Most of the ether-soluble peptides have been reported previously.[3] The acid-soluble fraction (P_3A) was obtained as follows. After repeated ether extraction of the acidified solution, a great deal of the yellow color remained in the water phase and the ether phases were also yellow. The ether extracts were combined, concentrated and finally washed once with acidified water. The wash water was added to the acid phase and evaporated to dryness in a Craig evaporator.[9] The residue was extracted with dry acetone to free the DNP derivatives of salts. Two preparations of P_3A were made. P_3A-I was made from an aliquot of the neutral fraction equivalent to 0.64 g. of collagen and P_3A-II from an aliquot equal to 1.24 g. P_3A-I was resolved into four bands on 10-g. columns of Super-cel-pH 5.0 (20 × 80 mm.) by elution with ethyl acetate saturated with water and the slow band remaining at the top of the column was eluted with the same solvent containing 1% acetic acid. P_3A-II was fractionated on 400-g. columns of Super-cel-pH 5.0 (80 × 300 mm.) and only three fractions were eluted. After the columns were extruded, the two remaining bands were cut out and extracted with 2% sodium bicarbonate. The bicarbonate extracts were concentrated, acidified and then extracted with ethyl acetate. The fractionation of P_3A-I required 80 columns and a period of a month and that of P_3A-II—which was about twice the amount of P_3A-I—used four columns and was completed within two weeks. All the fractions of both preparations were further resolved on buffered Whatman No. 1 (0.2 M acetate, pH 4.0) with buffered t-amyl alcohol as solvent. The bands were treated in the same way as the sections from the extruded columns.

Identification of the DNP-Peptides.—The quantity of DNP-peptide isolated was determined from the optical density at 350 mμ using a Beckman quartz spectrophotometer. An aliquot—0.2 to 0.4 μmole—was usually hydrolyzed by refluxing in 0.5 ml. of triple glass-distilled 6 N hydrochloric acid for a period of four hours. These hydrolytic conditions were not suitable for DNP-valyl or DNP-hydroxyprolyl peptides. For the latter, 12 N hydrochloric acid was used[10] and for the former, the hydrolysis was done in sealed tubes at 105° for 16 hours. Extractions of the

(1) W. A. Schroeder, L. M. Kay, J. LeGette, L. Honnen and F. C. Green, THIS JOURNAL, **76**, 3556 (1954).

(2) The abbreviations and representations of amino acid sequences are those of E. Brand (*Ann. N. Y. Acad. Sci.*, **47**, 187 (1946)) and F. Sanger and H. Tuppy (*Biochem. J.*, **49**, 463 (1951)).

(3) T. D. Kroner, W. Tabroff and J. J. McGarr, THIS JOURNAL, **75**, 4084 (1953).

(4) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(5) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(6) J. C. Perrone, *Nature*, **167**, 513 (1951).

(7) G. L. Mills, *Biochem. J.*, **50**, 707 (1952).

(8) F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353 (1953).

(9) L. C. Craig, J. D. Gregory and W. Haussmann, *Anal. Chem.*, **22**, 1462 (1950).

(10) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).

hydrolysates were made with ether or ethyl acetate and the aqueous phases from the four-hour hydrolysates were hydrolyzed for a further 16 hours at 105° in sealed tubes. The hydrolysates of certain peptides of the basic fraction were not extractable by ether or ethyl acetate. This indicated arginyl peptides and the four-hour hydrolysates were heated for another 16 hours at 105° in sealed tubes. No attempt was made to separate the DNP-arginine from the amino acids prior to paper chromatography since no interference was found.

The DNP-amino acids obtained from the hydrolysates were identified by paper chromatography on Whatman No. 1 filter paper. The following solvent systems were used for the ether-soluble DNP-amino acids: (1) buffered *t*-amyl alcohol and buffered paper—0.2 *M* acetate, *p*H 4.0; (2) benzene–acetic acid–water (1:1:1);[8] (3) benzene–1% aqueous acetic acid (1:1).[11] For the acid-soluble DNP-amino acids, the *t*-amyl alcohol and *n*-butanol saturated with water[11] served adequately for identification.

The amino acids were identified by buffered filter paper chromatography[12] and the phenol–*p*H 12 system was extensively used. The molar ratios of the constituent amino acids were determined by densitometry[13] and ranged between 60–80% of the amount of DNP-peptide hydrolyzed. The "multiple dipping" procedure of Jepson and Smith[14] has been employed and results in saving of time and material. Arginine has been detected by the Sakaguchi reaction[14] and by paper ionophoresis[15] with phosphate buffer, *p*H 6.0 and $\mu = 0.01$.

Determination of C-Terminal Residues.—The hydrazinolysis method of Akabori, Ohno and Narita[16] as modified by Blackburn and Lee[17] has been used to determine the C-terminal amino acid of DNP-peptides. The method consists of heating a protein with anhydrous hydrazine whereby only the C-terminal amino acid residues are liberated as the free amino acids and the other amino acid residues are converted to hydrazides. About 1 μmole of DNP-peptide is heated for 3 hours at 120° with 0.5 ml. of anhydrous hydrazine. The excess hydrazine is removed in a vacuum desiccator over concentrated sulfuric acid. The residue is dissolved in a little water and applied to a column (10 × 50 mm.) of the carboxylic ion-exchange resin, Amberlite IRC-50, in the *H*-form. The column is then washed with 20 ml. of water to remove the free amino acid and the basic hydrazides are retained on the column. The effluent is evaporated to dryness and run against the hydrolysate of the untreated DNP-peptide on paper chromatograms. The amino acid present in the chromatogram of the hydrazine-treated DNP-peptide is the C-terminal amino acid. On addition of hydrazine to a dry DNP-amino acid or DNP-peptide, the solution is a deep burgundy red instead of the characteristic yellow color of DNP derivatives in solution. However, at the end of the reaction time, the solutions are pale yellow or colorless. Recovery experiments of micromole quantities of glycine, aspartic acid and glutamic acid under the above conditions yielded 40% glycine, 10% aspartic acid and 20% glutamic acid. Locker[18] has reported higher recoveries for the neutral amino acids (85%) and for aspartic acid and glutamic acid 20 and 40%, respectively, by heating at 100° for 8 hours. Arginine was shown to be completely retained on the resin and to be destroyed by hydrazine at the high temperatures. In spite of the great losses, the method is very suitable for qualitative end-group determination and especially useful in those instances where only a small amount of DNP-peptide is available.

Prior to the employment of the Akabori method, we had been using the technique of Turner and Schmerzler[19] for the determination of the C-terminal amino acids. The peptide is heated with acetic anhydride and pyridine in a sealed tube at 150° for two to three hours and the C-terminal amino acid is converted to a derivative of aminoacetone and is not found on the paper chromatogram of the hydrolysate of the treated peptide. The recoveries were very low and the method is not applicable when proline or hydroxyproline is the C-terminal residue since the imino acids are not decarboxylated.[20]

Results

The peptides which have been positively identified from three fractions of the partial acid hydrolysate of steer hide collagen are listed in Tables I–IV. The aromatic fraction was also studied and the only positive result obtained was the identification of DNP-phenylalanine. The presence of acidic peptides in the neutral fraction must be attributed to incomplete adsorption of these peptides on the IR-4B resin. The sequences of some of the peptides could not be determined because of the small amount of the material finally isolated in the pure state.

A greater number of peptides was isolated from P_3A-II than from P_3A-I, but the recoveries of certain peptides were lower. Higher recoveries of the slower moving DNP-peptides—ser-hypro-gly, gly-(hypro,pro)-gly and glu-hypro-gly—were obtained from the smaller columns in spite of the fact that less material was processed. The tetrapeptide, gly-(hypro,pro)-gly (P_3A-II), was finally separated from glu-hypro by paper ionophoresis and the amount isolated was only sufficient for composition and C-terminal amino acid studies.

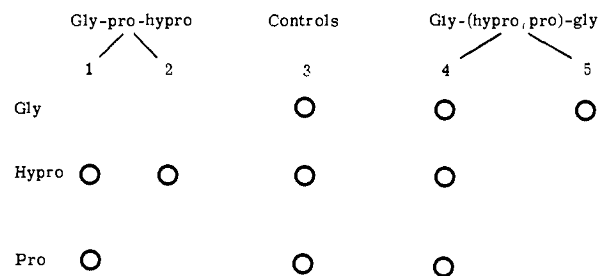

Fig. 1.—Diagram of paper chromatograms of two DNP-peptides developed with phenol–*p*H 12 on Whatman No. 1 buffered at *p*H 12. The acid hydrolysate of each peptide is shown in columns 1 and 4. The reaction product resulting from treatment of the respective peptides with anhydrous hydrazine appears in columns 2 and 5. Column 3 is the amido acid controls.

Figure 1 is a diagram demonstrating the determination of the sequence of gly-pro-hypro and the C-terminal amino acid of gly-(hypro,pro)-gly using anhydrous hydrazine. Prior to the employment of either the hydrazinolytic or the acetic anhydride–pyridine method, attempts to determine the sequences of these two DNP-peptides by partial acid hydrolysis had been unsuccessful. After refluxing the tripeptide with 6 *N* hydrochloric acid for 0.5 to 1 hr., only two bands were obtained on a paper chromatogram of the ether extracts. These were the DNP-tripeptide and DNP-glycine which was present to a greater extent. Because of the lability of the carboxyl group of glycine in peptide linkage, Sanger[21] recommends the use of 0.1 *N* hydrochloric acid to obtain partial hydrolysates of gly-

(11) E. F. Mellon, A. H. Korn and S. R. Hoover, THIS JOURNAL, **75**, 1675 (1953).
(12) E. F. McFarren, *Anal. Chem.*, **23**, 168 (1951).
(13) E. F. McFarren and J. A. Mills, *ibid.*, **24**, 650 (1952).
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(16) S. Akabori, K. Ohno and K. Narita, *Bull. Chem. Soc., Japan*, **25**, 214 (1952).
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(18) R. H. Locker, *Biochim. Biophys. Acta*, **14**, 533 (1954).
(19) R. A. Turner and G. Schmerzler, THIS JOURNAL, **76**, 949 (1954).
(20) H. D. Dakin and R. West, *J. Biol. Chem.*, **78**, 91 (1928).
(21) F. Sanger, *Biochem. J.*, **45**, 563 (1949).

TABLE I

PEPTIDES ISOLATED AS DNP DERIVATIVES FROM NEUTRAL FRACTION[a] OF PARTIAL HYDROLYSATE OF STEER HIDE COLLAGEN

	Acid-soluble P_3A[b]										
				%[f] of total amount of amino acid in collagen accounted for by amount of peptide isolated							
Peptide	Prepn.[c]	Agrees with -P-G-R-[d]	μmoles peptide/g. collagen[e]	Ala	Arg	Asp	Glu	Gly	Hypro	Pro	Ser
Ala-hypro	II	Yes	3.8	0.36					0.36		
Ala-hypro-gly	I	Yes	9.4	0.89				0.27	0.88		
	II		14.3	1.36				.41	1.33		
Ala-pro-gly	I	Yes	1.1	0.1				.03		0.08	
Glu-arg[1]	II	No	5.0		0.98		0.65				
Glu-hypro[g]	II	Yes	3.6				0.47		0.34		
Glu-hypro-gly	I	Yes	9.3				1.21	.27	0.87		
	II		6.9				0.90	.20	0.65		
Gly-asp-gly	II	No	1.9			0.40		.11			
Gly-glu-[g,1]	II	Yes	2.9				0.38	.08			
Gly-(hypro,pro)-gly	I	No	12.5					.71	1.17	.95	
	II		0.6					.03	0.06	.05	
Gly-pro-ala[1]	II	No	5.5	0.52				.16		.42	
Gly-pro-glu	I	No	0.9				0.12	.03		.07	
	II		2.9				0.38	.08		.22	
Gly-pro-gly	II	No	1.6					.09		.12	
Gly-pro-hypro	I	No	11.4					.33	1.07	.86	
	II		16.9					.48	1.58	1.28	
Ser-hypro-gly	I	Yes	5.7					.16	0.53		1.73
	II		2.2					.06	0.21		0.67
Ser-pro-gly	I	Yes	2.2					.06		0.17	0.67
	II		1.9					.05		0.15	0.58
Total[h]				2.3	1.0	0.4	3.1	2.6	6.2	3.2	2.4

[a] Fraction was obtained as the effluent after passage of the partial hydrolysate through columns of charcoal, cation and anion-exchange resins. [b] Separation is described in Experimental section. [c] Preparations I and II were made from aliquots of the neutral fraction equivalent to 0.64 and 1.24 g. of collagen, respectively. The latter was fractionated on 400-g. columns of Super-cel and the former on 10-g. columns. [d] -P-G-R- is the repeating sequence suggested by Astbury (*J. Intern. Leather Trades' Chemists*, 24, 69 (1940)) and used by Pauling and Corey (*Proc. Natl. Acad. Sci.*, **37**, 272 (1951)) where P represents prolyl or hydroxyprolyl, G glycyl and R one of the remaining amino acid residues. [e] Quantities of peptides are tabulated to the nearest 0.1 μmole of peptide. [f] The amino acid composition of steer hide collagen reported by Bowes and Kenten (*Biochem. J.*, **43**, 358 (1948)) has been used to calculate these percentages. [g] These peptides have also been found in the acid fraction. [h] If the same peptide was isolated from both preparations, the total includes the larger of the two amounts.

TABLE II

PEPTIDES ISOLATED AS DNP DERIVATIVES FROM NEUTRAL FRACTION[a] OF PARTIAL HYDROLYSATE OF STEER HIDE COLLAGEN

Ether-soluble			
Peptide	Agrees with -P-G-R-[b]	Peptide	Agrees with -P-G-R-[b]
Ala-gly-ala	No	Leu (ileu?)-ala	No
Gly-ala[1]	Yes	Ser-gly[1]	No
Gly-gly	No	Ser-hypro[c]	Yes
Gly-pro[1]	No	Thr-gly[1]	No
Hypro-gly[1]	Yes	Val-gly[1]	No

[a] Refer to Table I[a]. [b] Refer to Table I[d]. [c] This peptide was not reported previously (THIS JOURNAL **75**, 4084 (1953)).

cyl peptides. Hydrolysis of the tripeptide with the weak acid for 9 hours yielded the same two bands, but the DNP-tripeptide was recovered in a greater yield. Furthermore, compounds containing proline or hydroxyproline show a strong tendency to cyclize to form diketopiperazines.[22] Proline and hydroxyproline, but no peptide of the imino acids, were found in the partial hydrolysates. Likewise, not enough of the smaller peptides could be obtained from partial acid hydrolysates of the DNP-tetrapeptide for the determination of its sequence.

(22) E. L. Smith, "The Chemical Structure of Proteins," CIBA Foundation, New York, N. Y., 1953, p. 194.

TABLE III

PEPTIDES ISOLATED AS DNP DERIVATIVES FROM ACID FRACTION[a] OF PARTIAL HYDROLYSATE OF STEER HIDE COLLAGEN

Peptide	Agrees with -P-G-R-[b]	Peptide	Agrees with -P-G-R-[b]
Ala-asp[1]	No	Glu-gly[1]	No
Asp-asp	No	Glu-gly-gly	No
Asp(asp,gly)	No	Glu-hypro[c]	Yes
Asp-glu	No	Glu-met	No
Asp-gly	No	Glu-phe	No
Glu-ala[1]	No	Gly-glu[c,1]	Yes
Glu(ala,glu)	No	Leu (ileu?)-glu[1]	No
Glu(asp, gly)	No	Ser-asp	No
Glu-glu	No	Val-glu[1]	No

[a] This fraction was obtained by elution of a column of IR-4B in the acetate form at *p*H 3.5 with 0.6 *N* hydrochloric acid. [b] Refer to Table I[d]. [c] These peptides have also been found in P_3A-II.

It is probable that the order of the imino acids of the tetrapeptide is the same as in the tripeptide.

Discussion

The isolation and identification of the tripeptide, gly-pro-hypro, is the first unequivocal demonstration of the prolylhydroxyproline linkage in collagen. Dakin[23] reported the isolation of hydroxyprolylpro-

(23) H. D. Dakin, *J. Biol. Chem.*, **44**, 499 (1920).

TABLE IV

PEPTIDES ISOLATED AS DNP DERIVATIVES FROM BASIC FRACTION[a] OF PARTIAL HYDROLYSATE OF STEER HIDE COLLAGEN

Peptide	Agrees with -P-G-R-[b]	Peptide	Agrees with -P-G-R-[b]
Ala-arg[1]	No	Leu (ileu?)-arg	No
Arg-gly	No	Ser-arg[1]	No
Arg-gly-gly[1]	No	Val-arg	No
Arg-val-gly	No		

[a] The resin XE-64 buffered at *p*H 6.0 with ammonium acetate was eluted with *N* ammonium hydroxide to yield this fraction. [b] Refer to Table I[d].

line anhydride from a partial hydrolysate of gelatin. However, in order to obtain large yields of the anhydride, he extracted the partial hydrolysate with butanol under atmospheric pressure. Furthermore, Dakin states that these conditions favor the cyclization of proline with other amino acids. Gordon, Martin and Synge[24] indicated the probable presence of a dipeptide of proline and hydroxyproline in a partial hydrolysate of gelatin. It is likely that the tetrapeptide, gly-(hypo,pro)-gly, may have the sequence, gly-pro-hypro-gly, since none of the peptides found show a gly-hypro linkage. In Table I, the hypro-gly linkage is present in ala-hypro-gly, glu-hypro-gly and ser-hypro-gly. Furthermore, hypro-gly has been isolated in a rather high yield by Schroeder, *et al.*[1] Models of pro-hypro may be constructed easily and this sequence introduces an approximately 90°-change in the direction of the peptide chain.

Some indication of the lability of bonds can be inferred from this work. Schroeder, *et al.*, conclude that the carboxyl group of proline and the imino group of hydroxyproline are labile in peptide linkage because of the large amounts of gly-pro and hypro-gly found in the partial hydrolysates of gelatin and the failure to find the pro-hypro sequence. The isolation of ser-hypro, glu-hypro and most of the peptides recorded in Table I attests to the stability of the imino group of hydroxyproline and the carboxyl group of proline in the peptide bond. However, the conditions of hydrolysis may be responsible for the differences observed. Schroeder, *et al.*, hydrolyzed gelatin with 3.6 *N* hydrochloric acid for one week and fractionated the hydrolysate on Dowex 50-X8. The partial hydrolysate of collagen was obtained by the use of concentrated hydrochloric acid for four days and then fractionated mainly as the DNP derivatives. Nevertheless, fifteen dipeptides and two tripeptides isolated by Schroeder, *et al.*, are confirmed in this report. The lability of the peptide bond associated with the amino group of serine and threonine has been ascribed to N-acyl to O-acyl transformation[25] and thus in partial hydrolysates of proteins, ser-x and thr-x are found, but rarely x-ser and x-thr. This work indicates that the hydroxyl group of hydroxyproline does not react in the same way as the aliphatic hydroxyl group of serine and threonine. Furthermore, Gustavson[26] recently has proposed that the hydroxyl group of hydroxyproline is involved in interchain hydrogen bonding with the oxygen of the keto-imide linkage (-CO-NH-) and contributes to the stability of collagen. The aliphatic hydroxyl groups apparently are not involved in the stability of collagen since teleost collagen of higher aliphatic hydroxyamino acids content shows a lower shrink temperature than bovine collagen. The great number of dipeptides found in partial hydrolysates of collagen and gelatin may be a strong indication of the wide distribution of glycine and alanine residues throughout the peptide chain. The lability of peptides involving the residues of glycine and alanine linked through the -CO- or -NH- groups has recently been cited by Sanger.[27]

A total of 68 peptides has now been isolated from partial hydrolysates of collagen and gelatin[1,28,29] and only 15 agree with the sequence -P-G-R-. The following four peptides—ala-pro,[1] gly-asp,[1,29] leu (ileu?)-hypro[1] and lys-pro-gly[28]—in addition to those recorded in Tables I-IV agree with -P-G-R-. It is apparent from all reported work that about 20% of the peptides agree with -P-G-R- and this same trend is indicated by qualitative and quantitative data. Qualitative data have significance since the isolation of peptides under the conditions of fractionation employed should indicate a greater quantity in the protein. All the peptides reported in this work have been the major components of the various fractions and in no fraction investigated was there a major component not identified. It is to be noted that only two of the acidic peptides (Table III) and *none* of the basic peptides (Table IV) conform to the -P-G-R- sequence. Therefore, it would seem reasonable to conclude that the repetitive sequence -P-G-R- is not an essential requirement of the structure of collagen.

The recoveries of the peptides are low and may be attributed to incomplete dinitrophenylation and large losses which are encountered because of deficiencies in technique of fractionation. Recoveries from the paper fractionations have usually averaged about 40% and the column losses have not been determined. The values listed in Table I are minimal and undoubtedly the peptides were present to a much greater extent.

BEVERLY, MASS.

(24) A. H. Gordon, A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **37**, 92 (1943).

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(26) K. H. Gustavson, *Acta Chem. Scand.*, **8**, 1299 (1954).

(27) F. Sanger, *Advances in Protein Chem.*, **7**, 1 (1952).

(28) W. Grassmann and K. Riederle, *Biochem. Z.*, **284**, 177 (1936).

(29) K. Heyns, G. Anders and H. Becker, *Z. physiol. Chem.*, **287**, 120 (1951).