# THE GAMMA-GLUTAMYL BOND IN CHICK EMBRYO COLLAGEN

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# The $\gamma$-Glutamyl Bond in Chick Embryo Collagen

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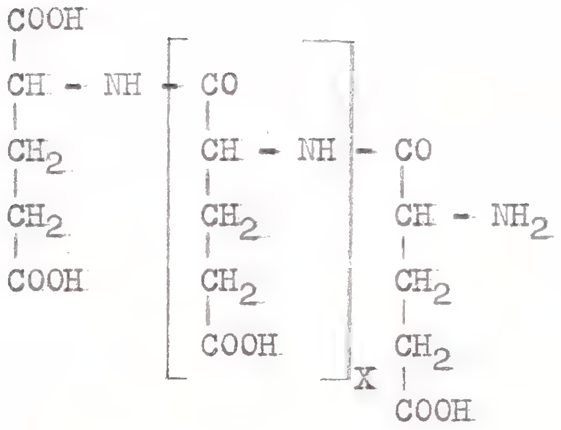
# Introduction

Quastel, Stewart, and Tunicliffe in 1923 (1) were the first to present evidence for the existence of gamma-glutamyl peptide linkages in a naturally occuring substance, glutathione, which had been isolated from yeast and demonstrated in various animal tissues by Hopkins in 1921 (2). Using the observation of Dakin (3) that alpha-amino acids, oxidized with hydrogen peroxide in the presence of a trace of iron salt, yield carboxylic acids with one carbon less than the original compound, Quastel et al. were able to oxidize impure preparations of glutathione with hydrogen peroxide and after acid hydrolysis to identify succinic acid among the products. This evidence suggested that the glutamic acid in glutathione was joined to the tripeptide through its gamma-carboxyl group leaving its alpha amino carboxylic grouping open to attack by the oxidizing agent. Kendall et al. (4) were able to demonstrate that oxidation of crystalline glutathione with hypobromite or Chloramine T did not disrupt the peptide bonds but did form products that yielded succinic acid on hydrolysis. The final proof for the presence of gamma-linkage in glutathione was offered by Harrington and Mead (5) who synthesized gamma-glutamylcysteinylglycine and showed that its

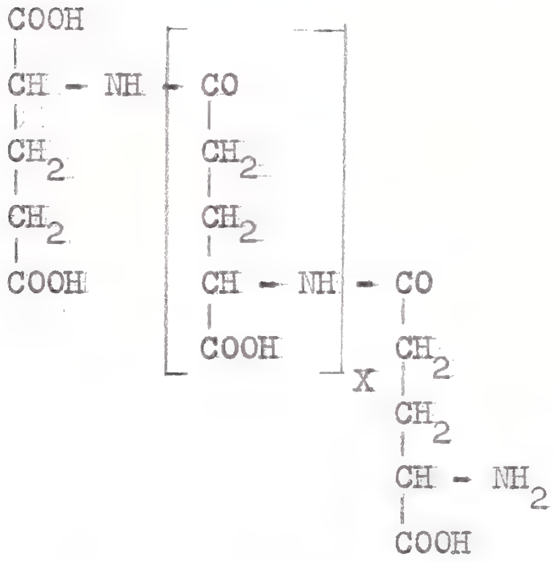
properties were identical with those of the crystalline peptide.

In 1937 Ivanovics and Bruckner (6) isolated and purified the capsular substance of Bacillus anthracis and Bacillus subtilis. On hydrolysis of the material they found only glutamic acid, but they were not able to say that the protein was made up solely of this amino acid. They pointed out that, if the capsular substance contained only glutamic acid, then two isomeric forms are possible depending on whether the alpha-carboxyl or the gamma-carboxyl or both are involved in the peptide linkages (Fig. 1).

Bovarnick (7) was able by comparison of total nitrogen to glutamic acid nitrogen to show that the capsular substance of Bacillus subtilis consisted solely of glutamic acid units. Working on the assumption that racemization of the glutamic acid units in alkaline solution would not occur if a free carboxyl was situated adjacent to the asymetric carbon atom (gamma-glutamyl form), Bovarnick allowed purified capsular substance to stand for ten days in alkaline solution. There was no change in optical rotation of the solution over this period - amino nitrogen showed only a small increase - from 10 to 20% - demonstrating that significant hydrolysis which would prevent racemization



Alpha-linkage

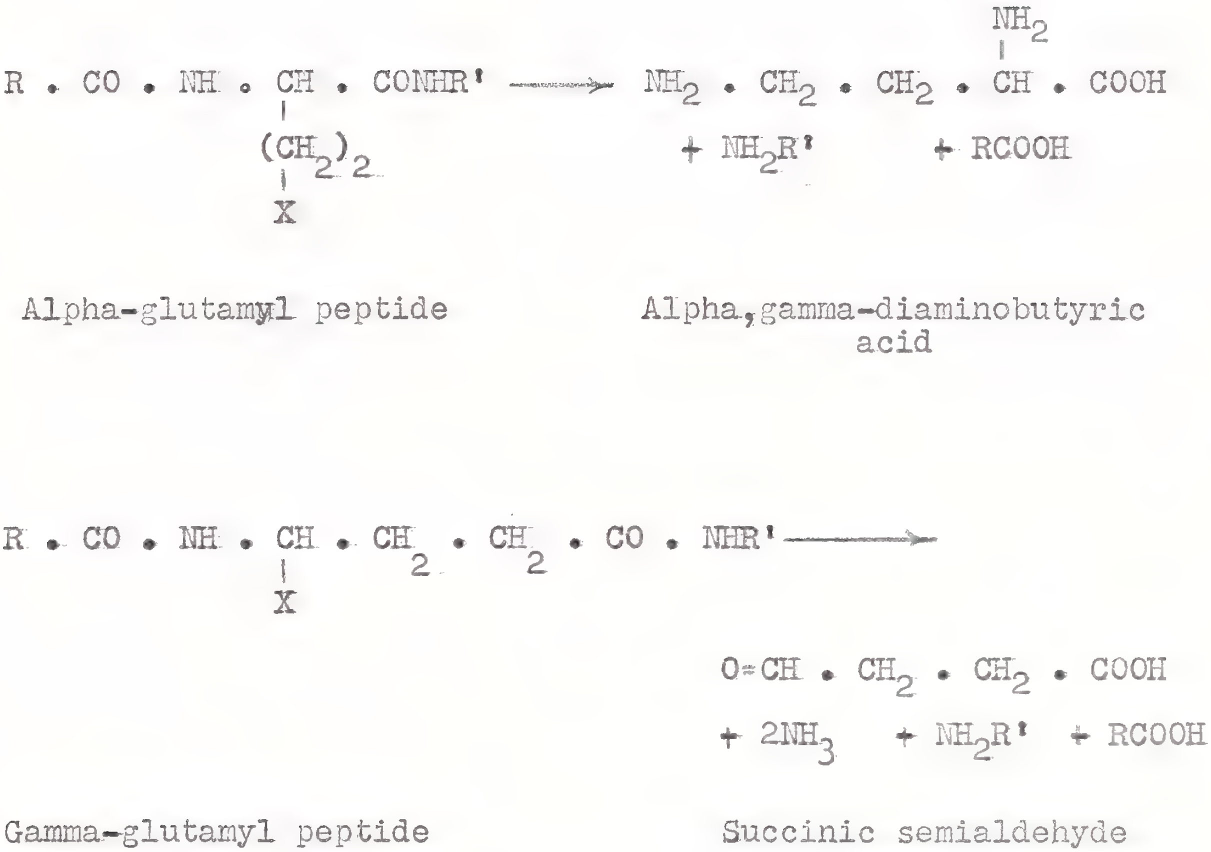


Gamma-linkage

Fig. 1. Structure of the alpha- and gamma-peptide linkages.

had not occurred. Gamma-peptide linkage of the glutamic acid was indicated on this basis. In support of this conclusion, a biuret test, which is specific for alpha-linked units, was negative.

Further evidence in support of a homogenous composition for the capsular substance was provided when Hanby and Rydon (8) converted the glutamic acid obtained from hydrolysis of *Bacillus anthracis* hapten to pyrrolidone-carboxylic acid and isolated this product quantitatively by chromatography on silica gel, thus verifying that the capsular substance was composed solely of glutamic acid units. The ready fission of the capsular substance by acid indicated to these investigators that the glutamic acid residues were linked, in part at least, by linkages which were more susceptible to acid hydrolysis than alpha-peptide bonds. They noticed that during Van Slyke amino-nitrogen assays the apparent amino nitrogen content of the capsular substance increased (the apparent molecular weight decreased) with increasing reaction time, a finding which indicated to the authors that in addition to the free amino group, the capsular substance also contained gamma-peptide groupings which liberated their nitrogen more slowly under Van Slyke conditions. One sample submitted to this procedure (molecular



$$R\cdot CO\cdot NH\cdot \underset{\underset{X}{|}}{\underset{(CH_2)_2}{\underset{|}{CH}}}\cdot CONHR' \longrightarrow NH_2\cdot CH_2\cdot CH_2\cdot \overset{NH_2}{\overset{|}{CH}}\cdot COOH + NH_2R' + RCOOH$$

Alpha-glutamyl peptide

Alpha,gamma-diaminobutyric acid

$$R\cdot CO\cdot NH\cdot \underset{\underset{X}{|}}{CH}\cdot CH_2\cdot CH_2\cdot CO\cdot NHR' \longrightarrow O{=}CH\cdot CH_2\cdot CH_2\cdot COOH + 2NH_3 + NH_2R' + RCOOH$$

Gamma-glutamyl peptide

Succinic semialdehyde

Fig. 2. Conversion of the alpha- and gamma-glutamyl peptides to the polyhydrazide followed by Curtius degradation. Unaltered polypeptide, $X{=}COOH$; polymethyl ester, $X{=}COOCH_3$; polyhydrazide, $X{=}CO\cdot NH\cdot NH_2$; product of Curtius degradation, $X{=}NH_3$

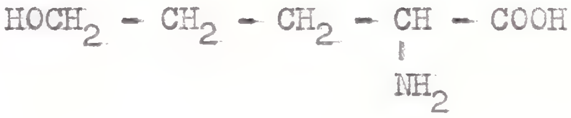
weight 5000) showed only a slight increase in amino-nitrogen with reaction time and they felt that this represented a final degradation product composed solely of alpha-peptide linkage, the gamma-linkages having been hydrolyzed by acid during the preparation.

Kovacs and Bruckner (9) (Fig. 2.) converted the poly-D-glutamic acid of <u>Bacillus subtilis</u> into the polymethyl ester and then into the polyhydrazide. After Curtius degradation followed by acid hydrolysis only succinic semialdehyde (the gamma-linkage rearrangement product) was found in the hydrolysate - there was no alpha,gamma-diaminobutyric acid. This indicated that in native poly-D-glutamic acid gamma-glutamyl bonds predominated. Later, Kovacs, Bruckner, and Kovacs (10) subjected alpha-L-polyglutamic acid hydrazide to the Curtius procedure and on hydrolysis recovered only alpha,gamma-diaminobutyric acid. Bruckner, Kovacs, and Nagy (11) then converted methyl poly-D-glutamate (prepared from the capsule of <u>Bacillus subtilis</u>) into the polyamide which was submitted to Hofmann degradation. After hydrolysis of the rearranged product only succinic semialdehyde was recovered.

Waley (12) synthesized Poly-(gamma-L-glutamyl)-L-glutamic acid. Many of its properties (eg. solubility in water; ionization constant, infra-red spectra, and reactivity to ninhydrin) were similar to those of <u>Bacillus subtilis</u> and

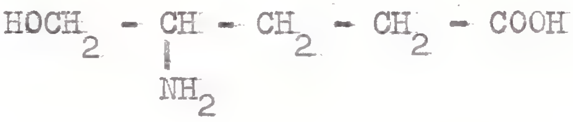
*Bacillus licheniformis* and differed from those of a synthetic alpha-linked polypeptide.

Chibnall, Rees, and Richards (13) esterified several samples of poly-glutamic acid from *Bacillus subtilis*, reduced them with lithium borohydride and analyzed the products given on subsequent acid hydrolysis. Under these conditions an alpha-linked glutamyl unit would be expected to yield delta-hydroxy-alpha-aminovaleric acid:



$$HOCH_2 - CH_2 - CH_2 - \underset{\displaystyle NH_2}{\underset{|}{CH}} - COOH$$

and a gamma-linked glutamyl unit would yield delta-hydroxy-gamma-aminovaleric acid:



$$HOCH_2 - \underset{\displaystyle NH_2}{\underset{|}{CH}} - CH_2 - CH_2 - COOH$$

Only the latter product was found, thus affirming the solely gamma-linked nature of the capsular substance. A synthetic poly-alpha-glutamic acid methyl ester treated in the same manner yielded 77.8% of total N as delta-hydroxy-alpha-aminovaleric acid and none as the gamma-linked reduction product.

Since the work on capsular substance was carried out, gamma-glutamyl linkages have been found in several natural

substances, a most interesting example being the presence of these bonds in collagen as ascertained by Gallop, Seifter et al (14).

These investigators noted that the conditions necessary to bring about Hofmann and Curtius rearrangements (references 9-11) when applied to proteins on a small scale, require drastic conditions and are often non-quantitative. In view of this, they studied the application of the Lossen rearrangement of the dinitrophenyl derivatives of hydroxamic acids to analysis of carboxyl groups in several compounds including a commercial base processed gelatin obtained from pig skin. Gallop et al. felt that the relative mildness of the conditions employed endowed the reactions with greater specificity. They esterified gelatin with methanol and acetic anhydride, formed the hydroxamic acid derivative by adding the methyl ester to neutral aqueous hydroxylamine, reacted the hydroxamate with FDNB[1] at pH 7.0 and promoted Lossen rearrangement of the hydroxamate-DNP derivative by heating at 100° C. for 2 min. in 0.1 N NaOH. After acid hydrolysis the rearrangement products, alpha,gamma-diaminobutyric acid in the case of alpha-linkage

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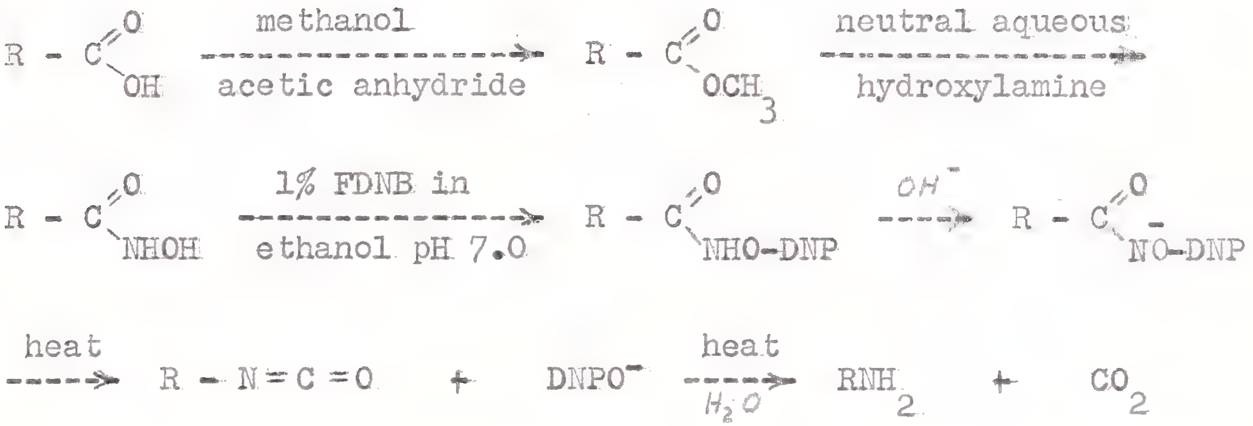
[1] The abbreviation used is: FDNB, fluorodinitrobenzene.

and succinic semialdehyde from gamma-linkage, were isolated (Fig. 3.). The presence of succinic semialdehyde was ascertained by forming the 2,4-dinitrophenylhydrazone and chromatographing against an authentic sample. Elution of the phenylhydrazone from the paper resulted in variable recoveries thereby limiting the procedure to a qualitative evaluation.

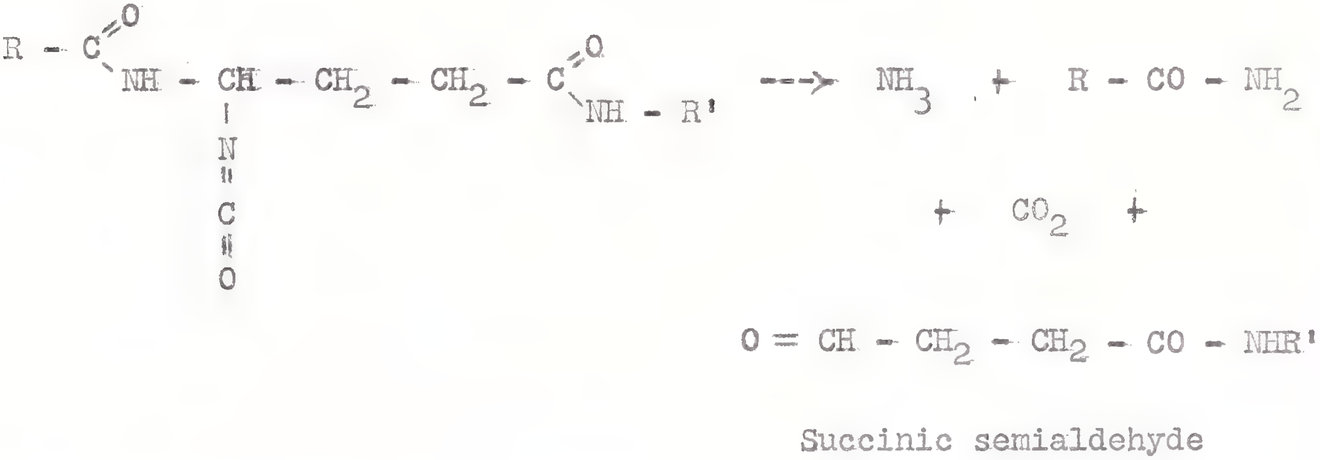
The authors note that the conditions of the procedure may promote interconversion of the alpha- and gamma-glutamyl peptide bonds. The esterification procedure was carried out in the presence of a dehydrating agent which could promote imide formation in the gelatin. Further, the gamma-glutamyl esters in the protein when undergoing attack by hydroxylamine under mild alkaline conditions might be converted to imide intermediates. In either case the imide intermediates could be cleaved with the formation of gamma instead of alpha peptide bonds (Fig. 4.). Thus the appearance of succinic semialdehyde in the hydrolysate of the rearranged gelatin preparation provided strong but not absolute evidence of gamma-linkage.

From their analysis of the hydrolysates of the pre- and post- rearranged gelatin, the authors observed that there was no serious discrepancy between the number of residues



$$R-C(=O)OH \xrightarrow[\text{acetic anhydride}]{\text{methanol}} R-C(=O)OCH_3 \xrightarrow[\text{hydroxylamine}]{\text{neutral aqueous}} R-C(=O)NHOH \xrightarrow[\text{ethanol pH 7.0}]{\text{1\% FDNB in}} R-C(=O)NHO\text{-}DNP \xrightarrow{OH^-} R-C(=O)\overset{-}{N}O\text{-}DNP \xrightarrow{\text{heat}} R-N=C=O + DNPO^- \xrightarrow[H_2O]{\text{heat}} RNH_2 + CO_2$$

The alpha-linked glutamic acid yields alpha,gamma-diaminobutyric acid after acid hydrolysis. The gamma-linked glutamic unit has a somewhat different fate, for each of these peptide bonds yields two molecules of ammonia, one from hydrolysis of the amine during Lossen rearrangement and another from subsequent acid hydrolysis of a C-terminal amide fragment, resulting in the appearance of succinic semialdehyde as the gamma rearrangement product.



$$R-C(=O)NH-CH(N=C=O)-CH_2-CH_2-C(=O)NH-R' \longrightarrow NH_3 + R-CO-NH_2 + CO_2 + O=CH-CH_2-CH_2-CO-NHR'$$

Fig. 3. Conversion of the gamma- and alpha- glutamic acid residues of gelatin to the hydroxamate, hydroxamate ester and Lossen rearrangement products.

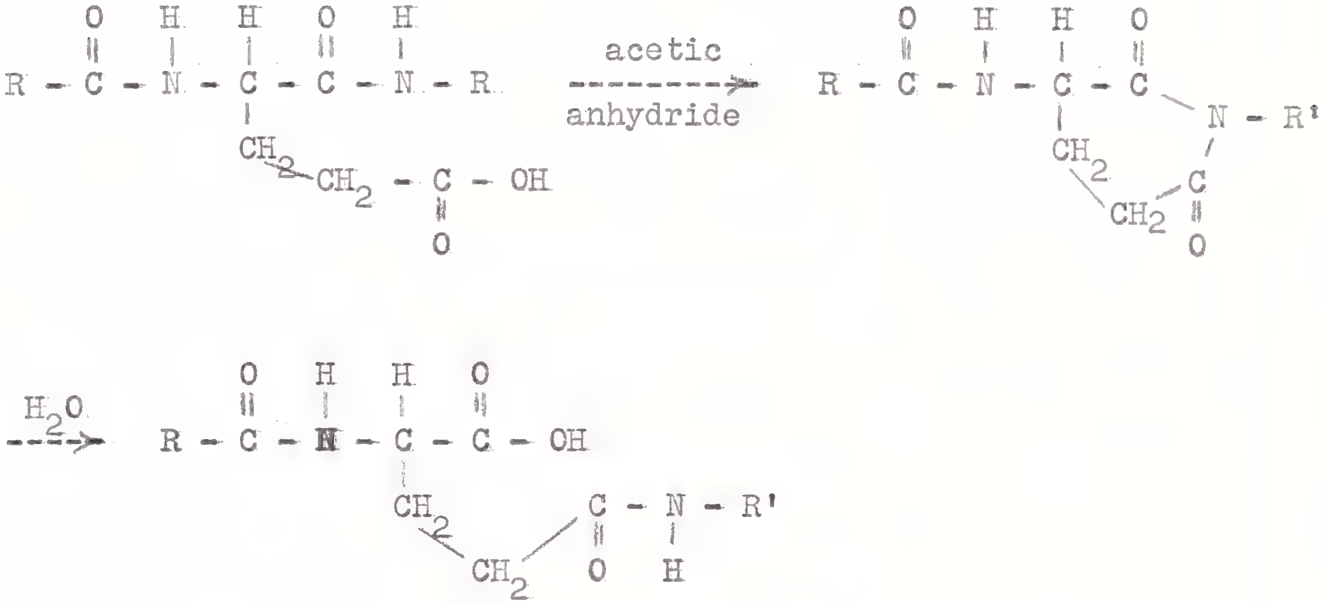


Fig. 4. Method of possible interconversion of alpha- and gamma-glutamyl peptide bonds in the presence of a dehydrating agent.

of aspartic acid which disappeared during Lossen rearrangement (13 residues per 1000) and the number of molecules of diaminopropionic acid which appeared (8.8 residues per 1000). However, the alpha,gamma-diaminobutyric acid recovered accounted for only 21% of the glutamic residues lost during rearrangement. They reasoned that, in the absence of theoretical contraindications, if interconversion of alpha- and gamma-bonds took place, then it should occur to an equal extent with both alpha-glutamyl and alpha-aspartyl linkages. On the basis of this reasoning they felt that some gamma-glutamyl peptide links existed in the original collagen.

Franzblau (15), in order to avoid conditions which could promote intermediate imide formation and possible alpha to gamma interchange and also to prevent hydroxylaminolysis of the intramolecular ester cross linkages (Gallop, Seifter, and Meilman (16)), performed a direct conversion by hydroxylamine hydrochloride of free carboxyl groups to hydroxamic acids by carrying out the reaction in aqueous, mildly acidic medium containing 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-p-toluene sulfonate, a water soluble carbodiimde described by Sheehan and Hlavka (17). Franzblau treated the hydroxamate derivative in the same manner

## Table I*

### Products identified after Lossen rearrangement of the dinitrophenyl hydroxamate derivative of gelatin derived from icthiocol as compared with a suitable control

(Expressed as residues per 1000 residues)

| Compound | Control gelatin | DNP-Hydroxamate Gelatin after Rearrangement | Difference |
|---|---|---|---|
| Glutamic acid | 71.4 | 46.0 | -25.4 |
| alpha, gamma-Diaminobutyric acid | 0 | 4.3 | +4.3 |
| Succinic semialdehyde | 0 | 22.0 | +22.0 |
| Ammonia | 42.0 | 78.0 | +36.0 |
| Aspartic acid | 45.8 | 39.6 | -6.2 |
| alpha, beta-Diaminopropionic acid | 0 | 5.0 | +5.0 |
| Dinitrophenol** | 0 | 31.0 | +31.0 |

* Franzblau, op. cit., p. 76.

** Each mole of dinitrophenylhydroxamate which undergoes rearrangement yields one mole of dinitrophenol.

as Gallop et al. except that the dinitrophenyl derivative of the hydroxamate was formed at pH 8.0 instead of pH 7.0. In this work the succinic semialdehyde was quantitated along with the other rearrangement products and with the amino acids obtained after acid hydrolysis of the rearranged gelatin (Table I). It is evident from Table I that per 1000 residues, 25 residues of glutamic acid were lost with rearrangement and 4.3 residues of alpha,gamma-diaminobutyric acid were recovered. If we are to assume that the rest of the unrecovered glutamic acid, (about 20 residues), was present as gamma-linked glutamic acid, then 40 residues of ammonia and 20 residues of succinic semialdehyde should be found per 1000 residues. In fact 36 molecules of ammonia beyond those present in the control gelatin and 22 molecules of succinic semialdehyde were recovered. Therefore Franzblau concluded that at least 20 residues of glutamic acid per 1000 residues of total amino acids in collagen are in gamma-glutamyl linkage. The failure of roughly half of the glutamate residues to be converted to hydroxamates does not affect Franzblau's quantitative statement; because he demonstrated in synthetic polypeptide experiments that the unconverted glutamic acid residues were in alpha-linkage. This conclusion was further supported by the fact that this

investigator had demonstrated the absence of alpha,gamma-carboxyl interchange when either alpha- or gamma-polyglutamic acid was subjected to an identical procedure. If a similar interpretation is applied to the aspartic acid residues then these must necessarily be alpha-linked.

In addition to the above results, obtained with gelatin prepared from icthyocol, Franzblau also carried out the same procedure with gelatin derived from calf-skin collagen. In this case, however, no quantitative estimation of the amount of succinic semialdehyde was carried out, although its presence was verified by chromatography.

In conjunction with some investigations being conducted in this laboratory in which a chick embryo system is being used to study the mechanism of formation of hydroxyproline from proline in the process of collagen synthesis, it was felt that it would be interesting to employ the same system in an investigation of the gamma-glutamyl bond. This dissertation will be concerned with the identification and measurement of gamma-linked glutamic acid in chick embryo collagen and with the establishment of an _in vivo_ preparation in which its formation may be studied.

## Materials

Bovine Achilles tendon collagen was purchased from the Worthington Biochemical Corp., Freehold, New Jersey. It had been purified by the method of Einbinder and Schubert (18).

Medium A was prepared according to Littlefield and Keller (19) and contained 0.25 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$, and 0.05 M Tris buffer, pH 7.6.

Sephadex-G-25 was obtained from Pharmacia, Uppsula, Sweden.

Dowex AG 50W-X8, 200-400 mesh, Hydrogen form, was purchased from the California Corporation for Biochemical Research, Los Angeles, California.

Silica gel G (according to Stahl) was purchased from Brinkmann Instruments Inc., Great Neck, New York.

1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-p-toluene sulfonate was purchased from the Aldrich Chemical Co., Milwaukee, Wisconsin. This compound will be referred to as WSC throughout the remainder of this paper. This is a water-soluble carbodiimide which Franzblau (15) found could promote, in an aqueous, slightly acidic medium, the direct conversion by hydroxylamine hydrochloride of free carboxyl to hydroxamic acid groups. When applied to the gelatin from icthyocol, Franzblau found that this method resulted in the conversion of 40 to 50% of the

free carboxyl groups to hydroxamic acids in 2 hours. A separate experiment conducted for 19 hours resulted in approximately 65% conversion to hydroxamic acid groups.

Acethydroxamic acid was prepared by a modification of the synthesis of benzohydroxamic acid according to Blatt (20). Separate solutions of 46.7 g. of hydroxylamine hydrochloride in 240 ml of methanol and 56.1 g. of KOH in 140 ml. of methanol were prepared at the boiling point of the solvent. Both solutions were cooled to 30-40° C., and the alkali solution was added to the hydroxylamine solution in an ice bath. This mixture was allowed to stand in an ice bath for 5 min., and the KCl which formed was filtered off on a Buchner funnel. 41 g. of ethyl acetate were added with stirring and the mixture was allowed to stand at room temperature for 6 hours. This solution was evaporated and an oil appeared which was neutralized to pH 7.0 with HCl. The neutralized oil was dissolved in hot ethyl acetate. The hot solution was allowed to cool slowly to 4° C. The acethydroxamic acid readily crystallized out on cooling. It was then filtered and stored in a dessicator *in vacuo*. The purity of the compound was verified by a melting point determination.

Succinic semialdehyde-2,4-dinitrophenylhydrazone was

prepared by the method of Hendler and Anfinsen (21). 147 mg. of glutamic acid were dissolved in 10 ml. of warm 0.1 N NaOH. Nitrogen was bubbled through the solution for a few minutes and 290 mg. of chloramine-T (sodio-p-toluenesulfochloramine) were added. The mixture was placed in a water bath at 50° C. for 15 min., cooled in ice, centrifuged and the insoluble p-toluenesulfonamide was discarded. To the supernatant solution containing succinic semialdehyde were added 28 ml. of 0.8% 2,4-dinitrophenylhydrazine in 2 N HCl. The suspension was filtered, and dried *in vacuo*. Purification of the compound was carried out by dissolving it in alcohol at 35° C., adding water at room temperature, and cooling in a dry ice-acetone mixture. The succinic semialdehyde-2,4-dinitrophenylhydrazone crystallized out on cooling; it was centrifuged, the supernatant was decanted, and the compound dried *in vacuo*. The M.P. of the prepared sample was 197-199° C.; the reported authentic M.P. is 199-201° C.

## Methods

*Preparation of cell-free enzyme fractions* - 15 7-day old White Leghorn chick embryos weighing 0.7 g. each were blended in three fourths of their volume of Medium A containing 10 umoles/ml of mercaptoethanol in a Servall Omni-Mix homogenizer for 15 seconds. The resulting suspension was spun for 10 minutes at 12,500 r.p.m. at 3° C. in the 40 rotor of the Spinco Model L centrifuge. The supernatant from this centrifugation was centrifuged as above for 20 min. at 15,000 r.p.m. The supernatant from this second centrifugation constituted the S-15 fraction. The S-15 fraction was centrifuged for 90 minutes at 40,000 r.p.m. (105 X g). The sediment from this S-105 fraction was homogenized in 15% of the volume of the S-105 fraction of Medium A with added mercaptoethanol as per above and this suspension constituted the microsomal fraction. To prepare the pH 5 enzyme system, the S-105 supernatant was brought to pH 5.2 with 1 *N* acetic acid, centrifuged for 15 minutes at 20,000 r.p.m., and the sediment was taken up in 15% of the S-105 volume of Medium A with added mercaptoethanol.

*Preparation of whole-cell incubations* - The chick eggs were shelled, the embryos were cut up with scissors, and minced in a loose-fitting glass homogenizer at slow speed

with either 2.5 or 3.0 ml of cold Krebs Ringer's phosphate solution per gram of tissue. Although a microscopic examination of the resultant suspension was not made, it was felt that this procedure yielded a predominantly whole-cell preparation.

Incubations were terminated by adding sufficient cold 10% or 20% TCA[1] to make a final concentration of 5%. In the case of the whole-cell preparations, the incubations were mixed thoroughly in a Waring blender (10 - 20 seconds) after precipitation of the protein.

<u>Extraction of the collagen as gelatin</u> - Collagen was separated from cold TCA-soluble material by washing the first precipitate 4 times with cold 5% TCA. The collagen was then solubilized in the gelatin form by heating in 5% TCA for 70 minutes at 90° C. after the method of Peterkofsky and Udenfriend (22). By this method these authors were consistently able to remove approximately 85% of the hydroxyproline from the precipitate. The hot-TCA extracts were extracted 4 times with anhydrous ether in order to remove the trichloroacetic acid.

<u>Purification of hot TCA-insoluble protein and determination of specific activity</u> - 4ml of 0.4*N* NaOH and

[1] The abbreviation used is: TCA, trichloroacetic acid.

0.5 ml of a solution of unlabeled glutamate (10 mg/ml) were added to the hot TCA-insoluble proteins and the solutions were heated at 60° for 2 minutes. 1.0 ml of 50% TCA was added to precipitate the proteins and the vessels were cooled in the refrigerator for 15 minutes. The vessels were centrifuged and the precipitated protein was washed two times with 95% ethanol and once with ether-ethanol solution (1:3 by volume). The protein was then heated to 70° C. for 2 minutes, washed once with anhydrous ether and dried. The dried purified protein was then dissolved in 1.0 ml of anhydrous formic acid and the activities of weighed amounts were determined.

Separation of the hot TCA-soluble fraction into a small molecular weight and a macromolecular fraction - The hot-TCA extracts were evaporated to a volume of 2.0 ml and placed on the top of columns of Sephadex G-25, 16 cm X 1.5 cm, containing 9.3 g dry weight of gel with a water regain of 2.4 g $H_2O$/g dry gel. The columns were eluted with 100 ml 0.2 M ammonium formate. 2 ml fractions were collected and alkaline hydrolysis according to the method of Hirs, Stein, and Moore (23) was carried out on each of the first 20 fractions eluted. 1.0 ml of 2.5 N NaOH was added to 0.13 ml aliquots of each 2.0 ml fraction to be hydrolyzed. The tubes were placed

in a 90° C. bath for 2.5 hours, during which time the contents evaporated to a volume of about 0.2 to 0.4 ml. The hydrolyzed fractions were then analyzed by a modified ninhydrin method. After 1.0 ml of 30%(by volume) acetic acid was added to each of the cooled tubes, in order to bring the solutions to about pH 5, 0.5 ml of ninhydrin reagent (prepared according to the method of Moore and Stein (24)) was added and the tubes were covered and placed in a boiling water bath for 15 minutes. After cooling, 2.5 ml of 1:1 (v/v) ethanol-water diluent were added to each tube and the absorbancy was read at 570 mμ against an ammomium formate standard. By this method the fractions containing the small molecular compounds were able to be identified by their respective ninhydrin reactive peaks. The macromolecular components came off the columns between about 11.0 ml and 16.0 ml. The smaller molecules started to be eluted at between 21.0 and 24.0 ml. The respective pooled macromolecular and small molecular fractions were lyophilized. The residue was dissolved in 1.0 ml of distilled water, and 0.1 ml and 0.2 ml samples were plated and counted.

All radioactivity was measured with a Nuclear-Chicago Geiger Counter, Model # 181 B.

Unless otherwise mentioned, acid hydrolysis was carried

out by making the solution of sample 6 N with respect to HCl and heating in a pressure cooker at approximately 15 lbs./sq. in., for three hours.

Preparation of the polyhydroxamic acid derivative of gelatin - This was carried out according to the method employed by Franzblau (15). 350 mg of hydroxylamine hydrochloride and 846 mg WSC were added per each 5.0 ml of aqueous gelatin solution (after evaporation of the gelatin solutions to convenient volumes). The resultant solutions were allowed to stand at room temperature, with occasional stirring, for 2.5 hours in the case of the bovine collagen and 2.0 hours in the chick embryo experiments. After standing, the protein was precipitated with cold acetone (care must be taken here since as much as one third of the collagen may not be precipitated), washed with ether and dried *in vacuo*. The dry hydroxamate derivative was then weighed and dissolved in varying volumes of warm distilled water as stated in the record of the individual experiments. The hydroxamic acid was either assayed by the procedure described at the end of the methods section of this paper or its presence was verified by placing a drop of the aqueous hydroxamate solution on a small quantity of ferric chloride in a test tube and observing the evolution of a wine-red color (the red reaction is specific for hydroxamic acid

groups since hydroxylamine itself does not yield color).

Dinitrophenylation and Lossen rearrangement of the gelatin polyhydroxamate derivative - The method employed was that of Seifter, Gallop at al (25) as modified by Franzblau (15). The pH of the aqueous hydroxamate solution was adjusted to 8.0 with NaOH and an equal volume of 1% FDNB (v/v) in ethanol was added with constant stirring by means of a magnetic bar. The pH of the mixture was measured with a pH meter and the reaction was maintained at pH 8.0 by constant titration with NaOH, until the pH was stable (approximately 5.0 minutes). The solution containing the hydroxamate-DNP derivative was extracted twice with anhydrous ether and once with petroleum ether in order to extract the excess fluorodinitrobenzene. The extracted solution was made 0.1 N with respect to NaOH and heated for 2 minutes at 100° C. to promote Lossen rearrangement.

Formation and extraction of the 2,4-dinitrophenylhydrazone derivatives - To either one half or all of the hydrolysate an equal volume of 0.8% 2,4-dinitrophenylhydrazine in 2 N HCl was added , and the resulting solution containing the 2,4-dinitrophenylhydrazones was evaporated to a convenient volume in a flash evaporator (the temperature of the evaporating bath was kept below

35° C.). The concentrated solution was extracted 2 or 3 times with ethyl acetate and the extracts were combined. The ethyl acetate solution was extracted with 0.1 M borate buffer, pH 9.0; until at least 2 extractions had been performed with the resulting borate solution being basic as tested by pH paper. The borate solutions were pooled and acidified with 2 N HCl and extracted into ethyl acetate. Where mentioned, this final ethyl acetate extract was further purified by electrophoresing a known volume on Whatman 3 MM paper with a sample of the standard succinic semialdehyde-2,4-dinitrophenylhydrazone at pH 8.6 in sodium barbital buffer (5 g sodium barbital, 3.25g sodium acetate trihydrate, and 34.2 ml of 0.1 N HCl brought to 1 liter with distilled water) at 1200 v across 59 cm for approximately 2 hours. The band corresponding to the marker was completely eluted with a known volume of 0.1 M borate buffer (pH 9.0).

Thin layer chromatography - It was found that paper chromatography as employed by both Gallop (14) and Franzblau (15) was totally inadequate in this work, as both the bovine and the avian experiments yielded several 2,4-dinitrophenylhydrazone derivatives which acted similarly on electrophoresis and one of these also migrated very close to the unknown on chromatography. After trying without success several methods of paper chromatography with a

great variety of solvents recommended for separation of 2,4-dinitrophenylhydrazone derivatives, it was found that a high degree of resolution with excellent separation of the components was attained by thin layer chromatography with silica gel. The 2,4-dinitrophenylhydrazones were developed by spraying with alcoholic KOH (3.5 ml 15 N KOH to 20 ml with ethanol). Glass plates were spread with Silica gel G, 0.5 mm in thickness, by conventional methods. These were allowed to stand at room temperature for a few minutes and then dried in an oven at 110° C.. The samples to be chromatographed were extracted from the borate buffer into the ethyl acetate as described above and a measured volume of the ethyl acetate was placed on the silica covered glass plates in a connected series of very small drops thus forming a line. Although it may not be necessary, the chromatograms were run in the dark. The solvent used for separation was tertiary amyl alcohol-ethanol-water (5:1:4, top layer). The band corresponding to succinic semialdehyde-2,4-dinitrophenylhydrazone was scraped off the plate into a test tube and eluted into a known volume of 0.1 M borate buffer, pH 9.0.[1] Part

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[1] It is interesting to note that in order to extract the succinic semialdehyde-2,4-dinitrophenylhydrazone from the silica gel with ethyl acetate, a drop of HCl must be added, a fact that suggests that, for an unknown reason, the carboxyl group is converted from the acid to the salt form during the process of chromatography. In any case, complete elution is readily attained with the borate buffer.

of this solution was assayed and part extracted into ethyl acetate and chromatographed with a sample of the authentic succinic semialdehyde-2,4-dinitrophenylhydrazone (the authentic sample had also been purified by chromatography in the tertiary amyl alcohol-ethanol-water system, eluted into borate solution, and extracted with ethyl acetate prior to its use as a standard marker) in the tertiary amyl alcohol-ethanol-water (5:1:4) solvent and also in a solvent of normal butanol saturated with water. In placing the experimental solution on the origin of the chromatogram, a line 3 cm long was made. The authentic marker was then placed in a similar series of dots forming a line 3 cm in length such that 1.5 cm of the marker was superimposed on the terminal 1.5 cm of the experimental line and 1.5 cm was free. Failure of the experimental sample to separate from the standard compound in two solvent systems was accepted as proof of identity.

<u>Desalting and isolation of amino acids</u> - The aqueous phase remaining after extraction of the hydrolysate with ethyl acetate was evaporated to a volume of two ml and placed on top of a 20 ml column containing Dowex 50-8X ($H^+$). The column was rinsed with 80 to 100 ml of distilled water and the amino acids were eluted with 50 ml of 2 N $NH_4OH$. The eluate was evaporated to 5.0 ml and known volumes

were placed on Whatman 3 MM paper and electrophoresed in pH 6.0 pyridine acetate buffer (100 ml pyridine and 10 ml glacial acetic acid brought to 1 liter with distilled water) at 3000 volts across 47 cm for 1 hour 15 minutes. Alpha,gamma-diaminobutyric acid was identified by its similar migration to a marker of the commercially prepared compound and by its characteristic red-brown staining reaction when the experimental marker section of the paper was sprayed with 0.5% ninhydrin in acetone. The area identified as alpha,gamma-diaminobutyric acid and those of the other amino acids desired were cut out and eluted from the electrophorogram with known volumes of water. These solutions were then assayed, and, when pertinent, their radioactivity was assesed.

Assay procedures - Hydroxamic acid assay was carried out according to the ferric perchlorate method of Seifter, Gallop et al (25). The solution (1 ml) containing 0.2 to 2.0 μmoles of hydroxamic acid was mixed with 2 ml of ferric perchlorate reagent (prepared by dissolving 0.8 g of pure iron wire in 10 ml of warm 60 % perchloric acid and bringing the solution to 100 ml with ethanol) diluted 1:1 with distilled water. After 5 minutes it was read at 505 mμ against a reagent blank. A standard curve was simultaneously prepared from a standard (2 μmoles/ml)

solution of acethydroxamic acid. This method measures only hydroxamic acids and gives no color from hydroxylamine. Optical density readings were performed with a Zeiss Spectrophotometer.

Glutamic acid and alpha,gamma-diaminobutyric acid were measured by a modified ninhydrin procedure as follows: Preparation of Ninhdrin reagent; 40 mg of $SnCl_2$ were dissolved in 25.0 ml pH 5.0 acetate buffer and 0.667g of ninhydrin were dissolved in 25.0 ml of methyl cellosolve (monomethyl ether of ethylene glycol). Just before the assay 8.3 ml of the $SnCl_2$ solution were mixed with all of the ninhydrin solution. Assay: to 0.5 ml samples, 0.5 ml pH 5.0 acetate buffer and 1.0 ml ninhydrin reagent were added in test tubes which had been rinsed several times with distilled water and dried. The solutions were mixed well, the test tubes were capped with glass marbles and they were placed in a covered boiling water bath for 15 minutes. At the end of this period they were cooled to room temperature in ice water and the volume was brought to 5.0 ml with 95% ethanol. The solutions were mixed well and the O.D. was read at 570 mμ against a reagent blank. Standard curves (0.05-0.50 μmoles) of glutamic acid and alpha,gamma-diaminobutyric acid were carried out each time the assay was done.

Hydroxyproline was measured according to the method of Newman & Logan (26) as modified by Leach (27) at 1/10 volume. To the sample brought to 0.1 ml with water was added 0.1 ml of 2.5 N NaOH. The tubes were placed in a 40° C. water bath, and when the contents of the tubes reached 40° C. (3-5 minutes), 0.1 ml of 6% $H_2O_2$ was added and the solution was mixed. After another 10 minutes in the bath, the solutions were cooled and 0.4 ml of 3 N $H_2SO_4$ and 0.2 ml of 5% p-dimethylaminobenzaldehyde in n-propanol were added. The tubes were placed in a 70° C. bath for 20 minutes, cooled and the O.D. was read at 555 mμ. Three different concentrations of each unknown solution were assayed and a standard curve (0.01 - 0.05 μmoles) was constructed with each assay.

Proline was measured by the method of Chinard (28). To 1.0 ml of the sample to be assayed, 1.0 ml of glacial acetic acid and 1.0 ml of ninhydrin reagent were added (each ml of reagent contained 0.4 ml of 6 N $H_3PO_4$ and 0.6 ml of glacial acetic acid; 25 mg of ninhydrin were added per ml of this acid mixture and the mixture was heated to about 70° C. to insure solution of the ninhydrin). The tubes were capped and heated at 100° C. for 1 hour, after which time they were cooled to room temperature, and brought to a volume of 5.0 ml with glacial acetic acid. The O.D. was read at 515 mμ against a reagent blank. A standard

curve (0.02-0.2 µmoles) was constructed and 3 different concentrations of each unknown solution were assayed.

Succinic semialdehyde was assayed according to the procedure described by Waelsch (29). The solution to be assayed was brought to 5.0 ml in borate buffer pH 9.0, and mixed with 2 ml of alcoholic KOH (3.5 ml of 15 N KOH made to 20 ml with absolute ethanol). After mixing the tube was placed in a water bath at 25° C. Exactly 2 minutes after addition of the alkali the absorbancy was measured at 420 mµ against a blank containing 5 ml of borate buffer and 2 ml of alcoholic KOH. A standard curve was simultaneously prepared from a standard solution (0.1 µmoles/ml) of the authentic compound in borate buffer.

## Results

### I. Incorporation of $C^{14}$-glutamic acid into the collagen-containing hot trichloroacetic acid-extractable fraction by a cell-free system from 7-day chick embryos.

Cell-free enzyme fractions were prepared from 15 7-day old White Leghorn chick embryos and incubated as described in Table II. The incubations were terminated by precipitating the protein with cold TCA. The precipitated protein was then washed, 20 µmoles of unlabeled glutamic acid carrier were added to each vessel, and the protein was extracted for 80 minutes with hot TCA. The hot TCA-extracts were extracted with ether, and their radioactivity was measured. That protein which was not soluble in hot TCA was purified and a determination of its specific activity was made. The gelatin-containing hot TCA-extracts were separated into small molecular weight and macromolecular fractions, and the radioactivity of each was determined (Table III).

From the data presented in Table II it is evident that the cell-free 7-day old chick embryo incubation using a pH 5 precipitated enzyme system in conjunction with the microsomal fraction, incorporated $C^{14}$-glutamic acid into the collagen-containing hot TCA-extractable protein fraction. The amount of incorporation, however, was small. The spec-

## Table II

### Incorporation of $C^{14}$-glutamic acid into the collagen-containing hot TCA-extractable fraction by a cell-free system from 7-day chick embryos and specific activity of the purified non-collagenous protein.

All incubations contained in a final volume of 6.0 ml: 0.30 ml Tris buffer 1.0 M, pH 7.7; 0.12 ml ATP, 50 µmoles/ml; 0.12 ml magnesium acetate 0.3 M; 0.03 ml KCl, 1.0 M; 0.12 ml GTP, 2.5 µmoles/ml; 0.24 ml glutathione, 200 µmoles/ml; 0.33 ml sodium phosphoenolpyruvate, 100 µmoles/ml; 0.12 ml pyruvate kinase, 1 mg/ml; 0.12 ml ascorbic acid, 50 µmoles/ml; 0.15 ml cysteine, 2 µmoles/ml; 0.30 ml amino acid mixture; containing-L-proline, argenine, lysine, threonine, serine, leucine, phenylalanine, methionine, valine, asparagine, aspartic acid, isoleucine, histidine, tryptophan, alanine, tyrosine, and glycine at a concentration of 1.0 µmole/ml. 0.50 ml of $C^{14}$-glutamic acid (28µc/µmole, 2.24 X $10^6$ cpm/ml) was added to vessels 1 and2 before incubation and to vessel 3 after precipitation of the protein.

The incubation was carried out at 38° C. for 90 minutes.

| Vessel # | Contents | | | Total activity of collagenous protein | Specific activity of non-collagenous protein |
|---|---|---|---|---|---|
| | pH 5 fraction | Microsome fraction | S-15 fraction | (cpm) | (cpm/mg) |
| 1 (pH 5) | 1.20ml | 1.80ml | - | 1290 | 100 |
| 2 (S-15) | - | 1.80ml | 1.20ml | 781 | 36 |
| 3 (pH 5 control) | 1.20ml | 1.80ml | - | 693 | 32 |

## Table III

The radioactivity in the macromolecular and small molecular fractions separated by means of a Sephadex G-25 column.

| Fraction | Macromolecular activity (total cpm) | Small molecular activity (total cpm) |
|---|---|---|
| pH 5 & Microsomes | 59 | 219 |
| S-15 & Microsomes | 104 | 118 |
| pH 5 control | Background | 119 |

ific activities of the non-collagenous protein fraction verify the fact that the extent of incorporation was not impressive. Due to the low incorporation of the system, it was decided to employ whole-cell systems from minces of embryos in the future.

Only a fraction (about 20%) of the total hot TCA extract radioactivity was recovered after Sephadex fractionation (Table III). Much of the activity recovered was present in the small molecular fractions, but there was some incorporation into macromolecular protein (eg. collagen). In a larger incubation employing $C^{14}$-glutamic acid of a higher specific activity it is expected that a greater incorporation into the macromolecular fraction would take place.

## II. Evidence for the presence of gamma-glutamyl bonds in a commercially prepared collagen obtained from bovine Achilles tendon.

For the purpose of testing the rearrangement procedure and the methods of recovery of the rearrangement products, it was decided to submit some commercially prepared collagen to the various manipulations that will be employed with the chick embryo gelatin.

2.0 g of bovine Achilles tendon collagen were suspended in 50 ml of distilled water and autoclaved at 125° C. for

3 hours. As some tissue remained at the end of this procedure, 20 additional ml of distilled water were added to the above solution and it was heated again under the same conditions. The solution was cooled, centrifuged, and the gelatin-containing supernatant was decanted. One half of the gelatin solution was hydrolyzed and served as the unrearranged control. The other half of the gelatin solution was evaporated to 5.0 ml and the polyhydroxamate derivative was formed. The dry gelatin hydroxamate weighed 949.2 mg and was dissolved in 62.5 ml of warm distilled water. There were 781 µmoles of hydroxamic acid in the solution submitted to Lossen rearrangement. The polyhydroxamate-DNP derivative was formed, the rearrangement was promoted by heating at alkaline pH, and the solution was hydrolyzed.

The hydrolysates of both the rearranged and the unrearranged gelatins were divided into two equal parts. One half of each hydrolysate was evaporated to 10 ml, 1.0 ml of this volume was electrophoresed at pH 6.0 without previous desalting, and glutamic acid, alpha,gamma-diaminobutyric acid and hydroxyproline were eluted and assayed. An equal volume of 0.8% 2,4-dinitrophenylhydrazine in 2 N HCl was added to the other half of each hydrolysate and the 2,4-dinitrophenylhydrazone derivatives were extracted

## Table IV

Analysis of some of the products in the Lossen rearranged dinitrophenylhydroxamate derivative of gelatin derived from bovine Achilles tendon as compared with an unrearranged control gelatin.

(Expressed as total number of µmoles recovered)

| Compound | Rearranged | Control | Rearranged values adjusted to hydroxyproline recovered from Control | Difference |
|---|---|---|---|---|
| Hydroxyproline | 467 | 676 | 676 | - |
| Glutamic acid | 284 | 612 | 411 | -201 |
| alpha,gamma-diaminobutyric acid | 13.1 | 0 | 19 | +19 |
| Succinic semi-aldehyde | 15.9 | 4.4 | 23 | +19 |

with ethyl acetate. A portion of the final ethyl acetate solution after extraction with borate buffer was chromatographed andthe succinic semialdehyde-2,4-dinitrophenylhydrazone was identified and assayed. In this case the above compound was eluted from the silica gel in 2.0 ml of ethyl acetate to which had been added a drop of 2 N HCl.

It may be seen from Table IV that some succinic semialdehyde was present in the hydrolyzed gelatin that had not been rearranged. The loss of glutamic acid in the rearranged as compared to the unrearranged control is not accounted for by the appearance of an equivalent quantity of glutamic acid rearrangement products in the rearranged gelatin. Because of this discrepancy no quantitative statements concerning the amount of gamma-linked glutamic acid present in the bovine Achilles tendon collagen can be made. It is evident that Lossen rearrangement of glutamic acid units took place, as witnessed by the appearance of alpha,gamma-diaminobutyric acid (the alpha-linked rearrangement product) in the rearranged gelatin and not in the unrearranged control. The rearranged gelatin also yielded 5.2 times as much succinic semialdehyde (the gamma-linked rearrangement product) as was endogenously present in the control.

[illegible]

solution [illegible]

hydrolysate [illegible]

compound was eluted [illegible]

acetate to which [illegible]

It may be seen th[illegible]

[illegible] as present in the hydrolyzed gelatin [illegible]

been rearranged. The loss of glutamic acid [illegible]

[illegible] as compared [illegible]

counted for by the [illegible]

of glutamic acid [illegible]

gelatin, because of this [illegible]

substances concerning [illegible]

acid present [illegible]

as acid. It is [illegible]

glutamic acid [illegible]

presence of alpha, gamma- [illegible]

limited [illegible]

and not [illegible]

gelatin also yielded [illegible]

hydro[illegible]

[illegible]

## III. The application of the Lossen rearrangement to the hot TCA-extractable protein derived from 14 day old chick embryos after incubation with $C^{14}$-glutamate.

It was decided to do an isotopic experiment with a 14 day old chick whole-cell preparation in order to see if the gamma-linked rearrangement product could be isolated from this age embryo, and also to see if the preparation would incorporate radioactivity into gamma-linked glutamyl units. As this experiment was designed as a quick pilot experiment to indicate where future investigation should be focused, neither a rearrangement control nor a radioactivity incorporation control was included.

A whole-cell incubation was prepared by homogenizing 20 14 day old White Leghorn chick embryos weighing approximately 9.9 g each with 2.5 ml of Krebs Ringer's phosphate solution per gram of tissue. To the resulting suspension were added 1.2 ml of a solution of $C^{14}$-glutamic acid (3.8 X $10^6$ cpm/ml, 205 μcuries/μmole) and 0.9 ml of a solution of $C^{14}$-glutamic acid (3 X $10^6$ cpm/ml, 170 μcuries/μmole), the total activity being equivalent to 11 μcuries. This whole-cell suspension was incubated at 37° C. for 1.5 hours with constant agitation. After precipitation, the protein was washed with cold TCA and extracted into hot TCA. The gelatin-containing solution was dialyzed against distilled water at 4° C. for 3 hours with one change in

the external phase midway in the dialysis. The total radioactivity of the hot TCA extract decreased from 13,000 cpm before dialysis to 8,500 cpm after dialysis.

The dialyzed solution was evaporated to 10 ml and the polyhydroxamate derivative was prepared as before. The dry hydroxamate weighing 1.0590 g was dissolved in 20 ml of warm distilled water (ferric chloride test was positive for hydroxamic acid), and the polyhydroxamate-DNP derivative was formed. A fine yellow gelatin-hydroxamate-DNP precipitate formed while the FDNB was being added to the hydroxamate solution. Franzblau noted that this occassionally happened when the pH was not well titrated, and that this precipitation limited the extent of the dinitrophenylation of the hydroxamate. During the extraction of the excess FDNB with anhydrous ether, a laboratory accident occurred which resulted in the loss of much of the gelatin-DNP derivative. An attempt to extract the recovered material with petroleum ether was abandoned because a difficult emulsion formed. NaOH was added to the solution and Lossen rearrangement was promoted by heating - the gelatin went back into solution on heating. The rearranged solution was evaporated to a volume of 20 ml, made 6 N with respect to HCl and hydrolyzed at 110° C. for 16 hours in vacuo.

## Table V

A partial analysis of the pertinent products obtained from the Lossen rearrangement of the hydroxamate-DNP derivative of gelatin of 14 day old chick embryos.

| Compound | Total yield (µmoles) | Total activity (cpm) |
|---|---|---|
| Glutamic acid | not assayed | 195 |
| Hydroxyproline | 138.5 | not counted |
| alpha,gamma-diamino butyric acid | not assayed | 109 |
| Succinic semialdehyde | 3.3 | 0 |

The hydrolysate was filtered and the 2,4-dinitrophenylhydrazone derivative was prepared from the entire hydrolysate. The resulting solution was evaporated to a convenient volume for extraction, and the 2,4-dinitrophenylhydrazones were extracted in ethyl acetate. This solution was re-extracted and chromatographed in the usual manner. The presence of succinic semialdehyde was verified in both the tertiary amyl alcohol - ethanol - water and in the water saturated butanol solvents. The compound was assayed and its radioactivity was determined. A known volume of hydrolysate remaining after extraction was electrophoresed at pH 6.0, the hydroxyproline was eluted and assayed, and the glutamic acid was eluted and its radioactivity determined. The resolution in the area of the electrophorogram containing the dibasic amino acids was poor, probably because of the presence of a large amount of salt. Therefore the remainder of the sample which had not been electrophoresed was desalted and then electrophoresed at pH 6.0. A small amount of alpha,gamma-diaminobutyric acid was identified, eluted, and its radioactivity determined.

In this experiment succinic semialdehyde was isolated and identified. However, in the absence of an unrearranged control, one cannot state, at this juncture, that the gamma-

linkage must necessarily exist in gelatin derived from the chick embryo.

It is interesting to note from Table V that there was radioactivity present in the alpha,gamma-diaminobutyric acid and that none existed in the succinic semialdehyde. If the counts in alpha,gamma-diaminobutyric acid represent incorporation and if this succinic semialdehyde originated as a result of Lossen rearrangement of gamma-glutamyl bonds in gelatin, there appear to be three possible explanations for the absence of radioactivity from the succinic semialdehyde. First, this succinic semialdehyde might come from collagen already present in the embryos and not from newly synthesized collagen. However, this system is known to synthesize collagen actively, as Lukens (30) has found that a 14-day whole-cell system from chick embryos will incorporate labeled proline into hydroxyproline. As hydroxyproline, for all practical purposes in this instance, is only present in collagen, this incorporation serves as a proof of collagen synthesis. The second explanation that must be considered is that there is a delay between the incorporation of $C^{14}$-glutamate into alpha-linkage and its incorporation into gamma-linkage, such that a measureable amount of activity is not incorporated into the gamma

rearrangement product during the period of the incubation. This possibility will be discussed later. The third explanation is that the diaminobutyric acid may have been derived from non-collagenous protein, and the collagen may not be highly enough labeled.

The low activity presented in Table V for glutamic acid in relation to that of alpha,gamma-diaminobutyric acid may be due, in part, to the fact that all of the glutamic acid was plated for counting and that the solution was not desalted before electrophoresis, as was the alpha,gamma-diaminobutyric acid sample. Both of these factors would tend to increase self-absorption. As most of the gelatin-hydroxamate-DNP derivative was lost, these values represent only a small amount of the total activity of the original sample.

In any event, it may be stated that the 14-day chick embryo whole-cell system apparently incorporated $C^{14}$-glutamic acid into hot TCA-extractable protein, and that a small portion of this activity was recoverable, after Lossen rearrangement, as the alpha-linkage rearrangement product.

## IV. The presence of the gamma-glutamyl peptide linkage in the hot TCA-extractable protein of the 14-day chick embryo.

It was now decided to establish more rigorously whether or not the collagen-containing fraction of 14 day old chick embryos contained glutamic acid in the gamma-glutamyl linkage.

A whole-cell incubation was prepared from 20 14-day White Leghorn chick embryos weighing 13.8 g each with 3 ml of Krebs Ringer's phosphate solution per gram of tissue. The resulting suspension was incubated at 37° C. for two hours. The protein was precipitated, washed and extracted twice with 5% TCA. The two hot TCA extracts were combined and extracted with ether - during the ether extraction a white precipitate formed which was filtered out. The gelatin rich solution was dialyzed against distilled water at 4° C. for three hours with the external phase being changed every hour. This dialyzed solution was evaporated to 20 ml and redialyzed for 11 hours with one change of the external phase. The solution was then evaporated to 12 ml and the polyhydroxamate derivative was made. The hydroxamate derivative weighing 1.6157 g was dissolved in 106 ml of warm water and the solution was divided into two equal portions - one to be rearranged and one to remain as the unrearranged control. There were 463 µmoles of hydroxamic acid groups in the 52 ml submitted to Lossen rearrangement.

The control hydroxamate was hydrolyzed as usual after bubbling nitrogen through the sample for five minutes. The other half of the gelatin hydroxamate was subjected to

the Lossen rearrangement according to the usual procedure and was then hydrolyzed in the same manner as the control. The 2,4-dinitrophenylhydrazone derivative of each hydrolysate was made, extracted, electrophoresed and chromatographed. Succinic semialdehyde-2,4-dinitrophenylhydrazone was identified in the rearranged gelatin but not in the control. The identity of the compound was verified in two solvent systems, and it was assayed. After removal of the 2,4-dinitrophenylhydrazones, the aqueous phase was evaporated to 2.0 ml and desalted. After desalting, duplicate electrophoreses, elutions and assays were performed in order to demonstrate that the results obtained were not the result of differences in the degree of elution from the electrophorograms but represented real differences in the amino acid compositions of the control and rearranged gelatins.

In this experiment the presence of gamma-linked glutamic acid in the gelatin-rich hot TCA-extractable protein from the 14-day chick embryo was affirmed. From Table VI it is evident that there was a great deal less glutamic acid recovered from the rearranged gelatin than was recovered from the control. The fact that the amount of proline remained relatively constant, as indeed

## Table VI

Analysis of the products obtained on hydrolysis of the Lossen rearranged gelatin from the 14-day chick embryo as compared with a suitable unrearranged control.

| Compound | Total µmoles recovered | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control Gelatin | | | Rearranged Gelatin | | | Difference* |
| | Duplicates** | | Average | Duplicates | | Average | |
| Hydroxyproline | 235 | 240 | 238 | 228 | 240 | 234 | N.S.*** |
| Proline | 480 | 485 | 483 | 492 | 504 | 498 | +15 |
| Glutamic acid | 413 | 435 | 424 | 295 | 275 | 285 | -139 |
| Alpha, gamma-diaminobutyric acid | 0 | 0 | 0 | 8.34 | 9.34 | 8.84 | +8.84 |
| Succinic semialdehyde | 0 | | 0 | 12.21**** | | 12.21 | +12.21 |
| $\frac{\mu\text{moles Hypro}}{\mu\text{moles Succinic semialdehyde}}$ | - | - | - | 18.7 | 19.7 | 19.2 | - |

* Difference between control average and rearranged average.

** Duplicates = Duplicate elutions from duplicate electrophoreses.

*** N.S. = Not significant.

****A single isolation and assay was done with succinic semialdehyde.

it should for it is not involved in the rearrangement procedure, indicates that the decrease in glutamic acid units is real. The appearance of 21 µmoles of glutamic acid rearrangement products still leaves 118 µmoles unaccounted for out of the 139 µmoles of the glutamic acid that disappeared as a result of the rearrangement. As the alpha,gamma-diaminobutyric acid was isolated from the same electrophorograms in the same way as the other amino acids that were analyzed, and as there is no evidence that this compound is unstable, it would not seem as though the discrepancy can be explained in terms of any large amounts of alpha,gamma-diaminobutyric acid being unaccounted for. It seems likely that the failure to account for the missing residues is due to the incomplete recovery of succinic semialdehyde as the phenylhydrazone. If the discrepancy lies in the unrecovered succinic semialdehyde-2,4-dinitrophenylhydrazone, then it probably lies in the loss of succinic semialdehyde during hydrolysis, the incomplete formation of the hydrazone derivative, or in incomplete extraction procedures. Precautions against photic degradation or heat breakdown of the hydrazone derivative were observed during the experimental procedure. Because of the aforementioned discrepancy, an absolute statement of the number of gamma-linkages in gelatin cannot be made. However, one

may conclude that there is at least one glutamic acid residue in gamma-peptide linkage for every 19 hydroxyproline residues in the gelatin-containing fraction derived from the 14-day chick embryo.

It might be worth repeating at this point that these investigations were focused on the establishment of an *in vivo* preparation in which the gamma-glutamyl bond formation could be studied. Therefore it is not quantitative recovery of succinic semialdehyde that is important but rather the attainment of a high degree of purity for this compound.

## Discussion

Two of the questions that are raised by the presence of gamma-glutamyl linkages are that of how and when in the process of protein synthesis, as we conceive of it today, the bonds are formed. It is hoped that the work reviewed in the experimental section of this paper will, with greater refinement, lead to the establishment of a whole-cell system which may be used as a tool with which to study the enzyme or enzymes responsible for the formation of this gamma linkage.

Figure 5. is a partial representation of the mechanism of protein synthesis as it is conceived of today. There are several stages in the synthetic process where the formation ofthe gamma-linkage may take place. First it may take place at the level of the formation of the aminoacyl-s-RNA complex. It is known from Ochoa (31) that the aminoacyladenylate-enzyme complex interacts with the corresponding transfer RNA whereby the amino acid is esterified through its carboxyl group to a hydroxyl residue on the terminal adenosine moiety of transfer RNA.

In the formation of the gamma-glutamyl bond, it may be that the glutamic acid attaches to the s-RNA by its gamma-carboxyl group instead of by its alpha-carboxyl

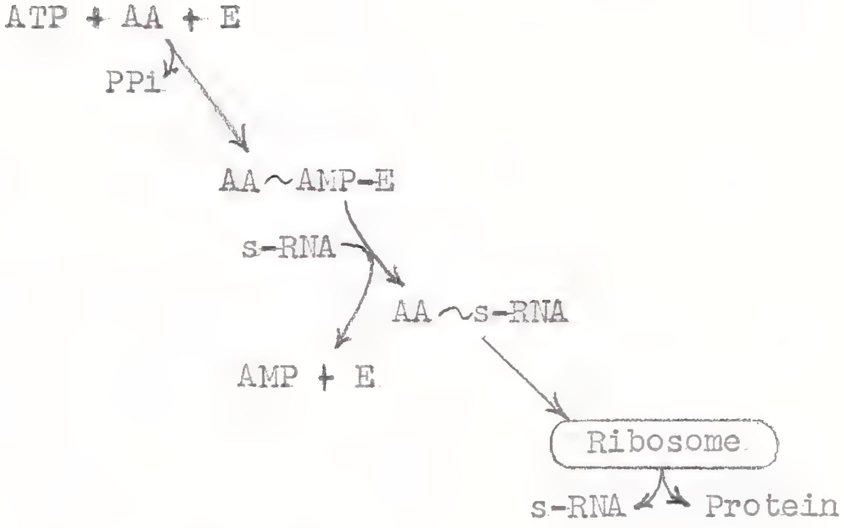

Fig. 5. Diagram of Protein Synthesis. E = enzyme, AA = amino acid, PPi = inorganic pyrophosphate, s-RNA = transfer RNA.

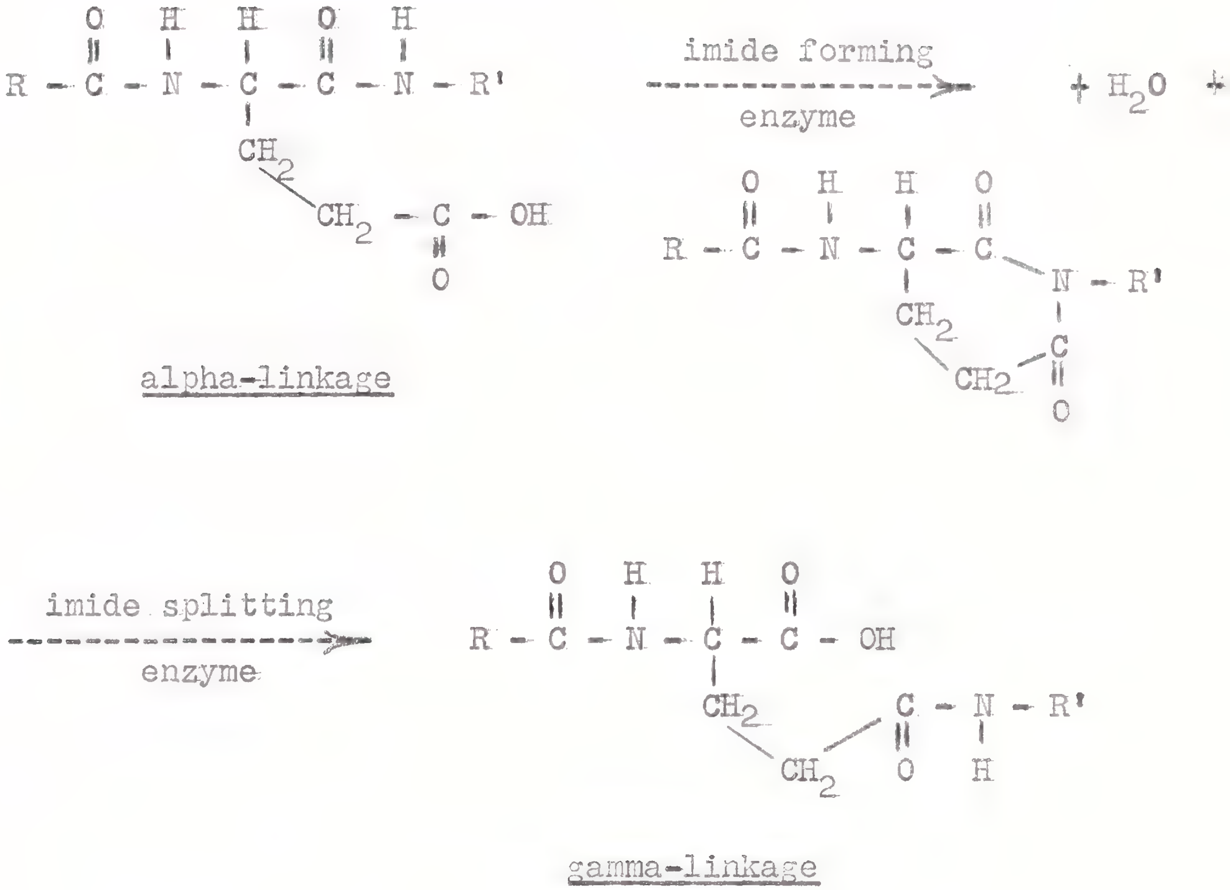

Fig. 6. Possible mechanism for intramolecular formation of gamma-glutamyl bonds.

group. In the formation of the usual peptide linkage there is a specific distance between the carboxyl group-s-RNA bond and the amino group which is entering into peptide linkage with the emerging protein. If we are to assume the formation of a glutamyl-gamma-carboxyl-s-RNA, then we must assume that the increased amino group to carboxyl group distance is accounted for either by folding of the amino acid or by bending of the s-RNA. If one is to assume that it is at this level that the gamma-linkage is formed, then it may be necessary to assume that there is a specific s-RNA for the glutamic acid entering gamma-linkage with a different code triplet or triplet order from the s-RNA of the glutamic acid entering into alpha-linkage.

A second possibility is that the gamma-linkage may be formed by an intramolecular rearrangement of the alpha-linked amino acid. Such a rearrangement could take place before or after polymerization of collagen subunits into tropocollagen. The assumption in this case is that glutamic acid would have been incorporated into alpha-peptide linkage, an imide might then be formed and finally the alpha-carboxyl-nitrogen bond would be hydrolyzed leaving the gamma-carboxyl group in peptide linkage. This would be the same type of reaction that was discussed in the introduction in relation to the possibility of

artificial formation of the gamma-linkage during the synthesis of the hydroxamic acid derivative of gelatin by the method of Gallop, Seifter et al (14).

If the formation of alpha- and gamma-linkages occurred at the same rate, as they presumably would if the gamma-bond is defined at the aminoacyl-s-RNA level, then one would expect the incorporation into gamma-linkage to show a similar variation with time as the incorporation into alpha-linkage. The same result would be expected if an intramolecular rearrangement took place in a short enough time after the incorporation of glutamic acid into the alpha-linkage, such that the delay period could not be determined. If, however, intramolecular rearrangement occurs and there is a significant delay between incorporation into alpha-linked glutamic acid and its rearrangement into the gamma-linked form, then one might expect that the alpha-linked glutamyl units would contain radioactivity before activity appeared in the gamma-linked units. This is a possible explanation for the presence of activity in the alpha,gamma-diaminobutyric acid and not in the succinic semialdehyde in experiment III.

Further experimentation will be focused on the study of the rate of incorporation of radioactivity into the

alpha- and gamma-linkages, in order to gain some insight into the nature of the enzyme system responsible for the gamma-bond formation. If it is shown that the bond is created by an intramolecular rearrangement, then I believe that this would be the first instance in which a peptide bond was demonstrated to be created in this manner.

## Summary

1) It was found that a cell-free system from 7-day old chick embryos would incorporate $C^{14}$-glutamic acid into the gelatin-rich hot-TCA-extractable protein. After separation of macromolecular from small molecular components, some of the activity was recovered in the macromolecular fraction. The amount of incorporation in this system was small.

2) Commercial bovine Achilles tendon collagen was converted to the polyhydroxamic acid-DNP derivative and submitted to Lossen rearrangement. Analysis of the hydrolysis products revealed the presence of succinic semialdehyde (the gamma-glutamyl-linkage rearrangement product) in both the rearranged gelatin and in an unrearranged control. The rearranged gelatin yielded 5.3 times as much succinic semialdehyde as it would be calculated to yield on the basis of the endogenous amount recovered from the unrearranged control. This collagen appears to contain at least 19 μmoles of gamma-glutamyl residues per 612 total glutamyl residues or per 676 residues of hydroxyproline. As discussed in the text, this is a minimal figure.

3) A whole-cell system of 20 14-day old chick embryos was incubated with $C^{14}$-glutamic acid. After conversion

to the polyhydroxamate-DNP derivative, the hot-TCA-extractable protein was submitted to Lossen rearrangement. Succinic semialdehyde was recovered and found to contain no radioactivity, whereas the alpha,gamma-diaminobutyric acid (alpha-linkage rearrangement product) which was recovered contained a small amount of radioactivity.

4) The collagen-containing hot-TCA-extractable protein was extracted from 20 14-day embryos. After formation of the polyhydroxamate derivative, one half of the solution was rearranged and the other was used as an unrearranged control. Analysis of the hydrolysis products revealed that both alpha- and gamma-linkage rearrangement products were present in the rearranged gelatin hydrolysate but not in that of the control. Quantitative analysis revealed that there was at least one gamma-linked glutamic acid residue for every nineteen hydroxyproline residues in the chick embryo collagen-containing hot TCA-extract.

5) A discussion of the possible mechanisms of gamma-bond formation was presented.

## Bibliography

1. Quastel, J.H., Stewart, C.P., and Tunnicliffe, H.E., Biochem. J. 17, 586 (1923).
2. Dakin, H.D., J. Biol. Chem. 1, 171 (1905).
3. Hopkins, F.G., Biochem. J. 15, 286 (1921).
4. Kendall, E.C., Mason, H.L., and McKenzie, B.F., J. Biol. Chem. 87, 55 (1930).
5. Harrington, C.R., and Mead, T.H., Biochem. J. 29, 1602 (1935).
6. Ivanovics, G., and Bruckner, V., Zeitschrift für Immunitatsforschung und Experimentelle Therapie. 90, 304 (1937).
7. Bovarnick, M., J. Biol. Chem. 145, 415 (1942).
8. Hanby, W.E., and Rydon, H.N., Biochem. J., 40, 297 (1946).
9. Kovacs, J., and Bruckner, V., J. Chem. Soc. 4255 (1952).
10. Kovacs, J., Bruckner, V., and Kovacs, K., J. Chem. Soc., 145 (1953).
11. Bruckner, V., Kovacs, J., and Nagy, H., J. Chem. Soc., 149 (1953).
12. Waley, S.G., J. Chem. Soc., 517 (1955).
13. Chibnall, A.C., Rees, M.W., and Richards, F.M., Biochem. J. 68, 246 (1958).

14. Gallop, P.M., Seifter, S., Lukin, M., and Meilman, E., J. Biol. Chem. 235, 2619 (1960).

15. Franzblau, C., "Unusual Linkages in Collagen", Ph.D. Thesis, Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University,. Obtained from University Microfilms, Inc., Ann Arbor, Michigan.

16. Gallop, P.M., Seifter, S., and Meilman, E., Nature 183, 1659 (1959).

17. Sheehan, J.C., and Hlavaka, J.J., J. Org. Chem. 21, 439 (1956).

18. Einbinder, J., and Schubert, M., J. Biol. Chem. 188, 335 (1951).

19. Littlefield, J.W., and Keller, E.B., J. Biol. Chem. 224, 13 (1957).

20. Blatt, A.H., in "Organic Synthesis" 7, 67 (1927).

21. Hendler, R.W., and Anfinson, C.B., J. Biol. Chem. 209, 55 (1954).

22. Peterkofsky, B., and Udenfriend, S., Biochem. and Biophys. Res. Comm. 6, 184 (1961).

23. Hirs, C.H.W., Moore, S., and Stein, W.H., J. Biol. Chem. 219, 623 (1956).

24. Moore, S., and Stein, W.H., J. Biol. Chem. 176, 367 (1948).

25. Seifter, S., Gallop, P.M., Michaels, S., and Meilman, E., J. Biol. Chem. 235, 2613 (1960).

26. Neuman, R.E., and Logan, M.A., J. Biol. Chem. 184, 299 (1950).

27. Leach, A.A., Biochem. J. 74, 70 (1960).

28. Chinard, F.P., J. Biol. Chem. 199, 91 (1952).

29. Waelsch, H. in "Methods in Enzymology", vol. III (S.P. Colowick and N.O. Kaplan, ed.) p. 573, Academic Press, New York (1957).

30. Lukens, L., Personal Communication of unpublished material.

31. Ochoa, S., in "Informational Macromolecules", (H.J. Vogel, V. Bryson, and J.O. Lampen, ed.) p. 4, Academic Press, New York (1963).