

T113  
+Y12  
2617

YALE UNIVERSITY LIBRARY



3 9002 06679 0867

THE GAMMA-GLUTAMYL BOND IN  
CHICK EMBRYO COLLAGEN

---

---

WILLIAM PRATT

1964

YALE



MEDICAL LIBRARY



Digitized by the Internet Archive  
in 2017 with funding from  
Arcadia Fund

<https://archive.org/details/yglutamylbondinc00prat>







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

By

William Brewster Pratt

B.A., Dartmouth College, 1960



A thesis Presented to the Faculty and Officers of  
the Yale University School of Medicine in partial  
fulfillment of the requirements for the degree of  
Doctor of Medicine

April, 1964

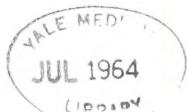
Department of Biochemistry  
Yale University School of Medicine

The Acquaintant Board in our community

WJ

336-2, delivered mail-in

MAIL DELIVERED AND RECEIVED A.S.



T113  
Y12  
2617

336-2, ring A

RECORDED TO INDEXED  
INDEXED TO FILE

Acknowledgement

The author would like to thank Dr. Lewis Lukens for his patient guidance and encouragement in both the experimentation and in the writing of the thesis.



## Table of Contents

Introduction.....	1
Materials.....	16
Methods.....	19
Results.....	32
Discussion.....	50
Summary.....	56
Bibliography.....	58



## 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 occurring 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



(2)

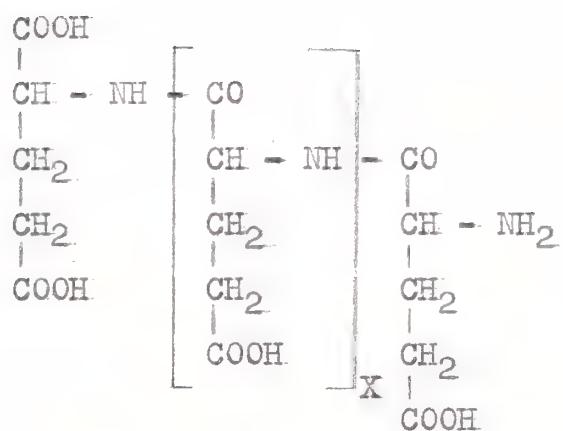
properties were identical with those of the crystalline peptide.

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).

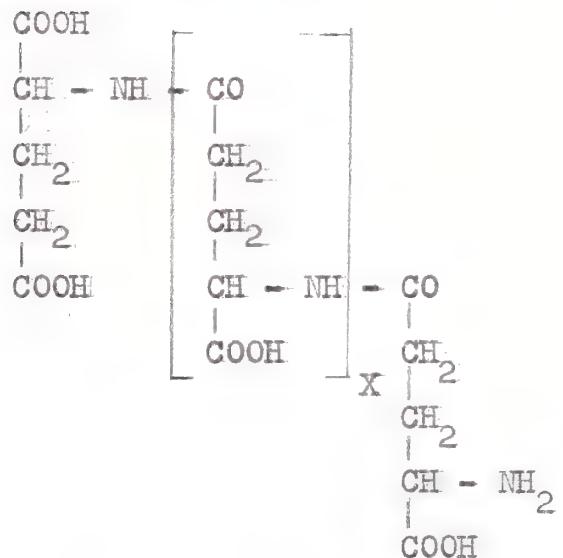
Bovarnick (7) was able by comparison of total nitrogen to glutamic acid nitrogen to show that the capsular substance of Bacillus subtilis consisted solely of glutamic acid units. Working on the assumption that racemization of the glutamic acid units in alkaline solution would not occur if a free carboxyl was situated adjacent to the asymmetric 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.



(3)



Alpha-linkage



Gamma-linkage

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

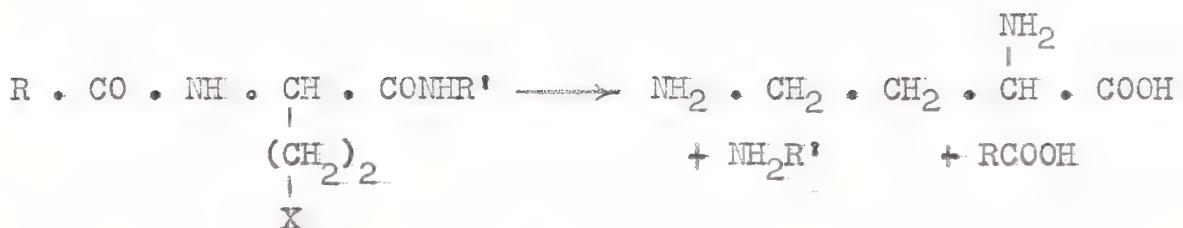


had not occurred. Gamma-peptide linkage of the glutamic acid was indicated on this basis. In support of this conclusion, a biuret test, which is specific for alpha-linked units, was negative.

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

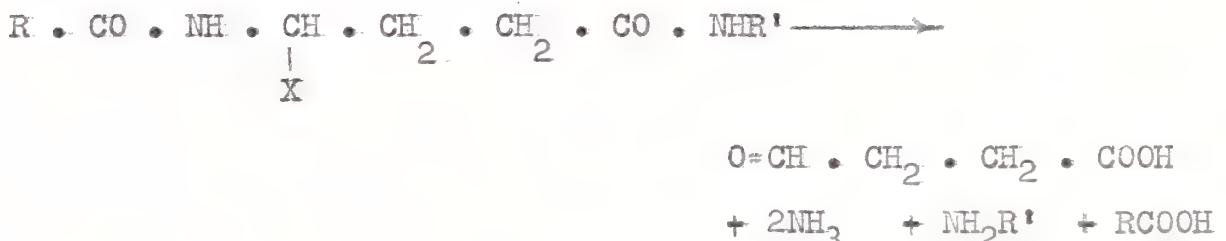


(5)



Alpha-glutamyl peptide

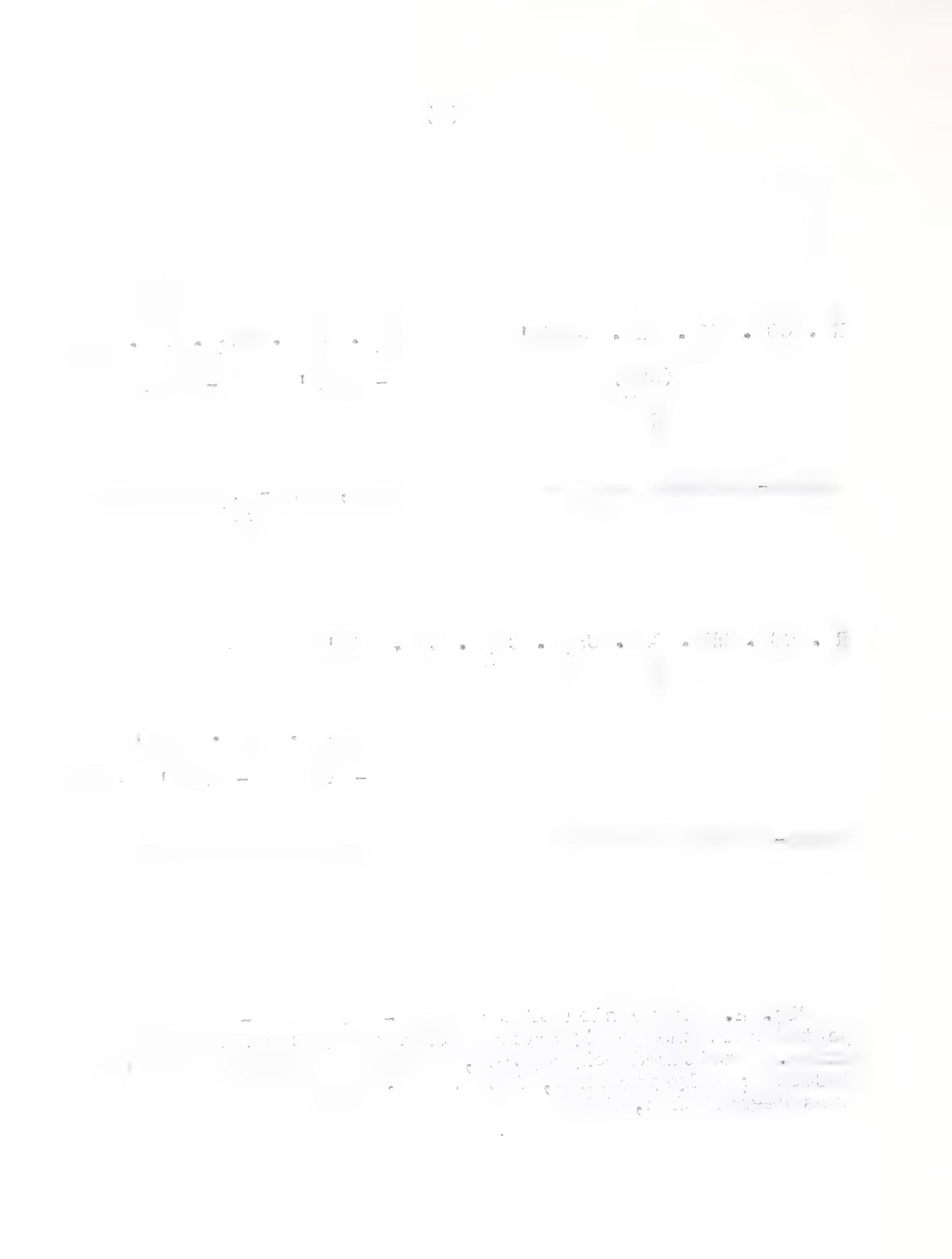
Alpha,gamma-diaminobutyric acid



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<sub>3</sub>; polyhydrazide, X=CO·NH·NH<sub>2</sub>; product of Curtius degradation, X=NH<sub>3</sub><sup>+</sup>



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 Bacillus subtilis 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 Bacillus subtilis) into the polyamide which was submitted to Hofmann degradation. After hydrolysis of the rearranged product only succinic semialdehyde was recovered.

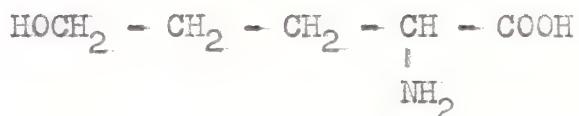
Waley (12) synthesized Poly-(gamma-L-glutamyl)-L-glutamic acid. Many of its properties (eg. solubility in water, ionization constant, infra-red spectra, and reactivity to ninhydrin) were similar to those of Bacillus subtilis and



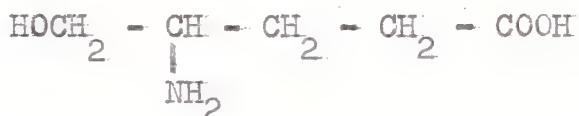
(7)

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:



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



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<sup>1</sup> 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

1. The abbreviation used is: FDNB, fluorodinitrobenzene.

the first time in the history of the world, the  
whole of the human race has been gathered  
together in one place, and that is the  
present meeting of the World's Congress.  
The first thing that I want to say is that  
I am very glad to see so many people here  
from all parts of the world, and I hope that  
they will all have a good time during their  
stay in this country. I also hope that they  
will be able to learn something new and  
interesting about our country and its people.  
I would like to say a few words about  
our country, which is called "India". India  
is a very large country, and it is the second  
largest country in the world. It is located in  
the southern part of Asia, and it has a  
population of over one billion people. The  
people of India are very friendly and  
welcoming, and they are always ready to  
help visitors. They speak English, and  
they are very interested in learning  
about other countries and their cultures.  
I hope that all the visitors will have a  
good time in India, and that they will  
be able to learn something new and  
interesting about our country and its people.

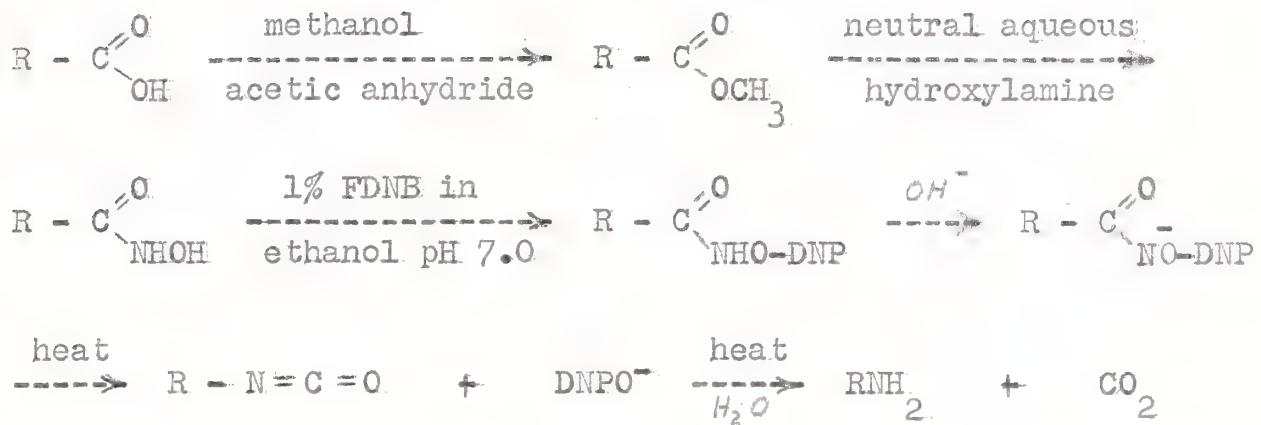
and succinic semialdehyde from gamma-linkage, were isolated (Fig. 3.). The presence of succinic semialdehyde was ascertained by forming the 2,4-dinitrophenylhydrazone and chromatographing against an authentic sample. Elution of the phenylhydrazone from the paper resulted in variable recoveries thereby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

the same time, the "flock" has been reduced to a mere remnant of its former size. The flock was originally composed of about 1000 birds, but now consists of only about 100 individuals. This reduction in numbers is due to a variety of causes, including the loss of habitat through urbanization and industrialization, as well as predation by introduced species such as the American Crow and the Red-tailed Hawk. In addition, the loss of food sources, particularly insects, has contributed to the decline in population. The future of the Common Grackle remains uncertain, as the species continues to face challenges from both natural and人为 factors.

(10)



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.

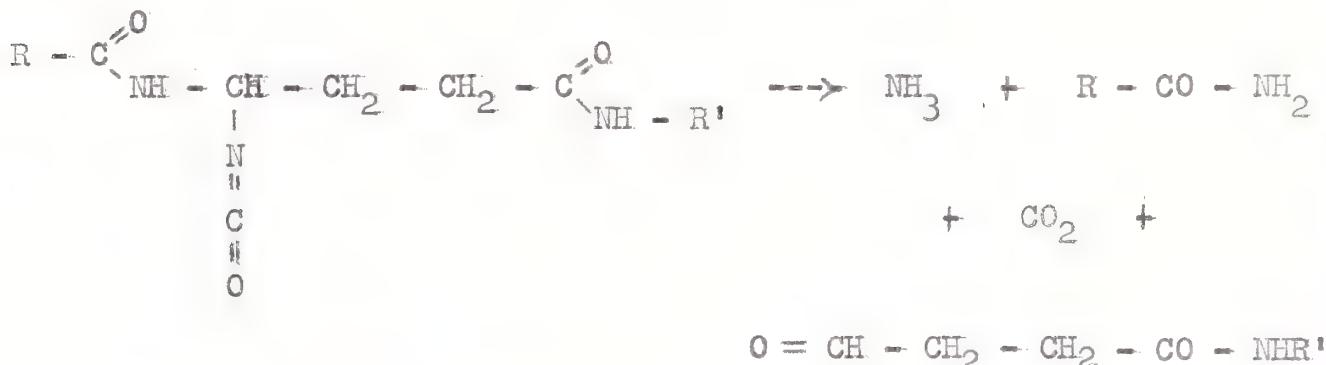


Fig. 3. Conversion of the gamma- and alpha- glutamic acid residues of gelatin to the hydroxamate, hydroxamate ester and Lossen rearrangement products.

the first time in the history of the world, the  
whole of the human race has been gathered  
together in one place, and that is the  
present meeting of the World's Fair.  
The great nations of the world have  
gathered here to exhibit their products,  
and to show the progress they have made  
in the arts and sciences. The United States  
is represented by a large number of  
exhibits, which are of great interest  
and value. The exhibits from other  
countries are also very interesting,  
and show the progress made by  
the different nations in various  
fields of endeavor. The exhibits  
are arranged in a very systematic  
manner, and are easily accessible  
to all visitors. The fair is a  
great success, and is a fitting  
tribute to the progress of  
the human race.

(11)

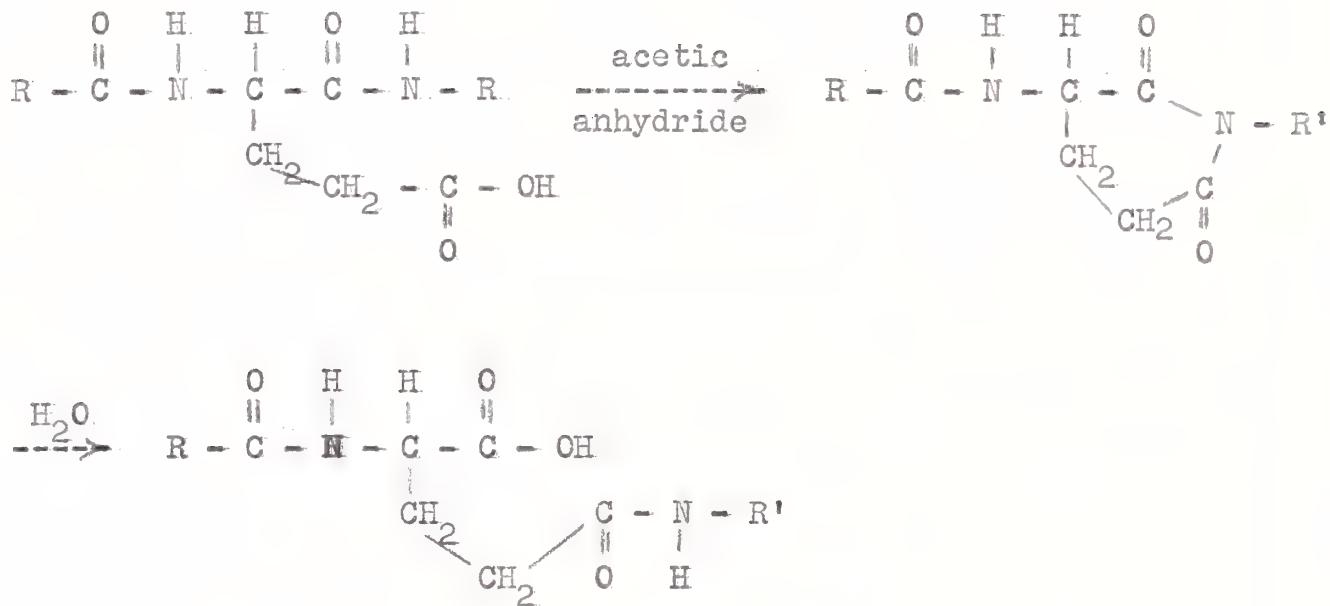


Fig. 4. Method of possible interconversion of alpha- and gamma-glutamyl peptide bonds in the presence of a dehydrating agent.

19

the first time in the history of the world, the people of the United States have been called upon to make a choice between two opposite ways of life, between two ways of living and of dying.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

The choice is between a republic and a monarchy; between a government of the people and a government of the few; between a government of the majority and a government of the minority.

(12)

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 carbodiimide 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 iethiocol as compared with a suitable control

(Expressed as residues per 1000 residues)

<u>Compound</u>	<u>Control gelatin</u>	<u>DNP-Hydroxamate Gelatin after Rearrangement</u>	<u>Difference</u>
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-Di- aminopropionic 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.

1. *U.S. Fish Commission*, 1871-1876  
2. *U.S. Fish Commission*, 1877-1881  
3. *U.S. Fish Commission*, 1882-1886  
4. *U.S. Fish Commission*, 1887-1891  
5. *U.S. Fish Commission*, 1892-1896  
6. *U.S. Fish Commission*, 1897-1901  
7. *U.S. Fish Commission*, 1902-1906  
8. *U.S. Fish Commission*, 1907-1911  
9. *U.S. Fish Commission*, 1912-1916  
10. *U.S. Fish Commission*, 1917-1921  
11. *U.S. Fish Commission*, 1922-1926  
12. *U.S. Fish Commission*, 1927-1931  
13. *U.S. Fish Commission*, 1932-1936  
14. *U.S. Fish Commission*, 1937-1941  
15. *U.S. Fish Commission*, 1942-1946  
16. *U.S. Fish Commission*, 1947-1951  
17. *U.S. Fish Commission*, 1952-1956  
18. *U.S. Fish Commission*, 1957-1961  
19. *U.S. Fish Commission*, 1962-1966  
20. *U.S. Fish Commission*, 1967-1971  
21. *U.S. Fish Commission*, 1972-1976  
22. *U.S. Fish Commission*, 1977-1981  
23. *U.S. Fish Commission*, 1982-1986  
24. *U.S. Fish Commission*, 1987-1991  
25. *U.S. Fish Commission*, 1992-1996  
26. *U.S. Fish Commission*, 1997-2001  
27. *U.S. Fish Commission*, 2002-2006  
28. *U.S. Fish Commission*, 2007-2011  
29. *U.S. Fish Commission*, 2012-2016  
30. *U.S. Fish Commission*, 2017-2021  
31. *U.S. Fish Commission*, 2022-2026  
32. *U.S. Fish Commission*, 2027-2031  
33. *U.S. Fish Commission*, 2032-2036  
34. *U.S. Fish Commission*, 2037-2041  
35. *U.S. Fish Commission*, 2042-2046  
36. *U.S. Fish Commission*, 2047-2051  
37. *U.S. Fish Commission*, 2052-2056  
38. *U.S. Fish Commission*, 2057-2061  
39. *U.S. Fish Commission*, 2062-2066  
40. *U.S. Fish Commission*, 2067-2071  
41. *U.S. Fish Commission*, 2072-2076  
42. *U.S. Fish Commission*, 2077-2081  
43. *U.S. Fish Commission*, 2082-2086  
44. *U.S. Fish Commission*, 2087-2091  
45. *U.S. Fish Commission*, 2092-2096  
46. *U.S. Fish Commission*, 2097-20100

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 ichthyocol, 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<sub>2</sub>, 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 ichthyocol, Franzblau found that this method resulted in the conversion of 40 to 50% of the

the first time in the history of the world, the whole of the  
population of the earth has been gathered together in one  
place, and that place is the city of New York.

The population of New York is now estimated at 2,500,000,  
and it is growing rapidly. The city is a great center of  
trade and commerce, and is the chief port of entry for  
the United States. It is also a great center of industry,  
and is the home of many large manufacturing concerns.  
The city is a great center of culture and learning, and  
is the home of many great universities and colleges.  
The city is a great center of art and music, and is the  
home of many great artists and musicians.  
The city is a great center of politics and government,  
and is the home of many great political leaders.  
The city is a great center of social life, and is the  
home of many great social organizations.  
The city is a great center of religion, and is the  
home of many great religious leaders.  
The city is a great center of science and research,  
and is the home of many great scientific leaders.  
The city is a great center of literature and writing,  
and is the home of many great literary leaders.  
The city is a great center of theater and drama,  
and is the home of many great theatrical leaders.  
The city is a great center of sports and recreation,  
and is the home of many great sports leaders.  
The city is a great center of fashion and style,  
and is the home of many great fashion leaders.  
The city is a great center of beauty and grace,  
and is the home of many great beauty leaders.  
The city is a great center of power and influence,  
and is the home of many great power leaders.  
The city is a great center of wealth and prosperity,  
and is the home of many great wealth leaders.  
The city is a great center of fame and glory,  
and is the home of many great fame leaders.  
The city is a great center of honor and respect,  
and is the home of many great honor leaders.  
The city is a great center of love and compassion,  
and is the home of many great love leaders.  
The city is a great center of hope and faith,  
and is the home of many great hope leaders.  
The city is a great center of courage and strength,  
and is the home of many great courage leaders.  
The city is a great center of wisdom and knowledge,  
and is the home of many great wisdom leaders.  
The city is a great center of beauty and grace,  
and is the home of many great beauty leaders.  
The city is a great center of power and influence,  
and is the home of many great power leaders.  
The city is a great center of wealth and prosperity,  
and is the home of many great wealth leaders.  
The city is a great center of fame and glory,  
and is the home of many great fame leaders.  
The city is a great center of honor and respect,  
and is the home of many great honor leaders.  
The city is a great center of love and compassion,  
and is the home of many great love leaders.  
The city is a great center of hope and faith,  
and is the home of many great hope leaders.  
The city is a great center of courage and strength,  
and is the home of many great courage leaders.  
The city is a great center of wisdom and knowledge,  
and is the home of many great wisdom leaders.

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

the first time in the history of the world, the  
whole of the human race has been gathered  
together in one place, and that is the  
present meeting of the World's Fair.  
The great number of people here  
from all parts of the world,  
and the great variety of things  
which are to be seen and heard,  
make it a most interesting and  
exciting place to visit.  
The exhibits are very numerous  
and varied, and include  
many valuable and interesting  
specimens of art and science.  
The buildings are large and  
handsome, and the grounds  
are well-kept and attractive.  
The atmosphere is friendly and  
welcoming, and the people  
are hospitable and kind.  
The food is good and plentiful,  
and there is something for  
every taste and preference.  
The entertainment is varied and  
interesting, and includes  
many performances by  
famous artists and performers.  
The whole experience is  
one of great pleasure and  
enjoyment, and is a fitting  
tribute to the progress and  
civilization of the human race.

prepared by the method of Hundler 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-toluene-sulfochloramine) 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.

the same time, the number of species per genus is much smaller than in the case of the *Phytolaccae*. The genera are more numerous, and the species more scattered, so that the number of species per genus is smaller. This is true of all the families, except the *Malvaceae*, which have a large number of species per genus. The *Malvaceae* have a large number of species per genus, but they are scattered among many genera.

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

the first time, in the year 1868, he was sent to the United States, and in 1870 he was appointed to the chair of History at the University of Michigan. He has written several books, among them "The History of the United States," "The History of the Civil War," "The History of the Slaveholding Kingdom," and "The History of the Slaveholding Kingdom." He is a member of the American Historical Association, and has been a member of the Board of Governors of the University of Michigan.

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<sup>1</sup> 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.

Extraction of the collagen as gelatin - 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.

Purification of hot TCA-insoluble protein and determination of specific activity - 4ml of 0.4N NaOH and

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

the first time in the history of the world, the whole of the  
population of the earth has been gathered together in one  
place, and that place is the city of New York.

It is a remarkable fact that the population of New York  
is greater than that of all the other cities in the United States  
combined. It is also a remarkable fact that the population of  
New York is greater than that of all the other cities in the world  
combined. This is a remarkable fact, and it is a fact that  
cannot be denied.

It is a remarkable fact that the population of New York  
is greater than that of all the other cities in the United States  
combined. It is also a remarkable fact that the population of  
New York is greater than that of all the other cities in the world  
combined. This is a remarkable fact, and it is a fact that  
cannot be denied.

It is a remarkable fact that the population of New York  
is greater than that of all the other cities in the United States  
combined. It is also a remarkable fact that the population of  
New York is greater than that of all the other cities in the world  
combined. This is a remarkable fact, and it is a fact that  
cannot be denied.

It is a remarkable fact that the population of New York  
is greater than that of all the other cities in the United States  
combined. It is also a remarkable fact that the population of  
New York is greater than that of all the other cities in the world  
combined. This is a remarkable fact, and it is a fact that  
cannot be denied.

It is a remarkable fact that the population of New York  
is greater than that of all the other cities in the United States  
combined. It is also a remarkable fact that the population of  
New York is greater than that of all the other cities in the world  
combined. This is a remarkable fact, and it is a fact that  
cannot be denied.

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<sub>2</sub>O/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

for the first time, and I am not at all sure that the best way  
to do it is to have a single large group of people who have  
been trained in one particular way. I think that the best way  
is to have a number of smaller groups, each with its own  
specialized training, and then to have them work together  
in a coordinated manner. This would allow each group to  
concentrate on its own area of expertise, while still being  
able to benefit from the knowledge and experience of other  
groups. It would also allow for more flexibility in terms of  
the types of projects that can be undertaken, as each group  
can focus on the specific needs of their clients. In addition,  
it would allow for more efficient use of resources, as each  
group can specialize in a particular area and therefore be  
more effective in addressing the needs of their clients. Finally,  
it would allow for more rapid growth and expansion, as each  
group can build on the success of the others and work together  
to achieve common goals.

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 m $\mu$  against an ammonium 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

the first time in the history of the world, the  
whole of the human race has been gathered  
together in one place, and that is the  
place where the people of all nations  
have come together to make a new  
constitution for the world. This  
is a great and wonderful thing, and it  
is a thing that has never been done  
before. It is a thing that has been  
done by the people of all nations,  
and it is a thing that has been done  
by the people of all races.  
It is a thing that has been done  
by the people of all creeds,  
and it is a thing that has been done  
by the people of all classes.  
It is a thing that has been done  
by the people of all ages,  
and it is a thing that has been done  
by the people of all countries.  
It is a thing that has been done  
by the people of all continents,  
and it is a thing that has been done  
by the people of all oceans.  
It is a thing that has been done  
by the people of all mountains,  
and it is a thing that has been done  
by the people of all plains.  
It is a thing that has been done  
by the people of all deserts,  
and it is a thing that has been done  
by the people of all forests.  
It is a thing that has been done  
by the people of all fields,  
and it is a thing that has been done  
by the people of all cities.  
It is a thing that has been done  
by the people of all towns,  
and it is a thing that has been done  
by the people of all villages.  
It is a thing that has been done  
by the people of all hamlets,  
and it is a thing that has been done  
by the people of all farms.  
It is a thing that has been done  
by the people of all ranches,  
and it is a thing that has been done  
by the people of all plantations.  
It is a thing that has been done  
by the people of all mines,  
and it is a thing that has been done  
by the people of all quarries.  
It is a thing that has been done  
by the people of all mills,  
and it is a thing that has been done  
by the people of all factories.  
It is a thing that has been done  
by the people of all workshops,  
and it is a thing that has been done  
by the people of all studios.  
It is a thing that has been done  
by the people of all studios,  
and it is a thing that has been done  
by the people of all studios.

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).

the same time, it is important to understand the basic principles involved and how they relate to the specific needs of the individual patient.

It is also important to understand the potential risks and benefits of different treatments, and to weigh these against the patient's overall health and life expectancy. This requires a careful assessment of the patient's medical history, current medications, and other factors that may affect their response to treatment. It is also important to consider the patient's preferences and goals, and to involve them in the decision-making process. This can help ensure that the chosen treatment is appropriate for the patient's individual needs and circumstances.

Finally, it is important to remember that cancer treatment is a complex and often challenging process. It requires a multidisciplinary team of healthcare professionals, including oncologists, nurses, and other specialists, working together to provide the best care possible. It also requires a commitment from the patient to follow through with the recommended treatment plan and to stay involved in their own care. By understanding the basics of cancer treatment and working closely with healthcare providers, patients can take an active role in their own health and well-being.

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 et 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-dinitrophenyl-hydrazone derivatives - To either one half or all of the hydrolysate an equal volume of 0.8% 2,4-dinitrophenyl-hydrazine 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

and the first time I have seen a specimen of the genus from an aquatic

habitat. It is a small, slender, elongated fish, with a compressed body.

The body is covered with numerous small scales.

The head is small, with a large mouth and a single dorsal fin.

The body is elongated and slightly compressed laterally, with a distinct

lateral line and a series of small scales along the sides.

The scales are small and closely set, giving the body a silvery appearance.

The fins are well developed and deeply forked, particularly the caudal fin.

The body is covered with numerous small scales.

The head is small, with a large mouth and a single dorsal fin.

The body is elongated and slightly compressed laterally, with a distinct

lateral line and a series of small scales along the sides.

The scales are small and closely set, giving the body a silvery appearance.

The fins are well developed and deeply forked, particularly the caudal fin.

The body is elongated and slightly compressed laterally, with a distinct

lateral line and a series of small scales along the sides.

The scales are small and closely set, giving the body a silvery appearance.

The fins are well developed and deeply forked, particularly the caudal fin.

The body is elongated and slightly compressed laterally, with a distinct

lateral line and a series of small scales along the sides.

The scales are small and closely set, giving the body a silvery appearance.

- 9 -

Specimen No. 12345

This is a small, slender, elongated fish, with a compressed body.

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 of 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

the first time in the history of the world, the whole of the human race has been gathered together in one place.

The second reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The third reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The fourth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The fifth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The sixth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The seventh reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The eighth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The ninth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The tenth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The eleventh reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The twelfth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The thirteenth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The fourteenth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The fifteenth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The sixteenth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The seventeenth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The eighteenth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The nineteenth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The twentieth reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The twenty-first reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The twenty-second reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

The twenty-third reason is that the world is now so small that it is possible for us to travel from one end of the globe to the other in a few days.

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.<sup>1</sup> Part

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.

# THE HISTORY OF THE AMERICAN PEOPLE

BY JAMES MCGOWAN  
PUBLISHED BY THE AMERICAN BOOK COMPANY  
AT NEW YORK AND BOSTON

IN TWO VOLUMES  
PRICE, \$1.50 EACH  
THE PAPER EDITION  
PRICE, \$1.00 EACH

THE PAPER EDITION  
CONTAINS 200 PAGES  
ADDED TO EACH VOLUME.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

THE PAPER EDITION  
IS PRINTED ON A  
SPECIALLY PREPARED  
PAPER, WHICH IS  
NOT LIKELY TO  
WORSEN WITH AGE.

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.

Desalting and isolation of amino acids - 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

and the first time I have seen it. It is a very large tree, about 100 ft. tall, with a trunk diameter of 10 ft. It has a very large root system, with many large roots extending out from the base. The bark is smooth and grey, with some lichen growing on it. The leaves are large and green, with a serrated edge. The flowers are small and yellow, with a sweet fragrance. The fruit is a small, round, red berry, with a sour taste. The tree is found in the forest, and is a valuable source of timber and fruit.

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 2000 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 assessed.

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  $\mu$ moles 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 m $\mu$  against a reagent blank. A standard curve was simultaneously prepared from a standard (2  $\mu$ moles/ml)

1. *Chlorophyllum* (L.) Pers. *Chlorophyllum* Pers.

2. *Cladonia* L. *Cladonia* L.

3. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

4. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

5. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

6. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

7. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

8. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

9. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

10. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

11. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

12. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

13. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

14. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

15. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

16. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

17. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

18. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

19. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

20. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

21. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

22. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

23. *Cochlidiochasma* (L.) Pers. *Cochlidiochasma* Pers.

solution of acethydroxamic acid. This method measures only hydroxamic acids and gives no color from hydroxyl-amine. 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  $\text{SnCl}_2$  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  $\text{SnCl}_2$  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  $\mu\text{m}$  against a reagent blank. Standard curves (0.05-0.50  $\mu\text{moles}$ ) 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>SO<sub>4</sub> 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 m $\mu$ . Three different concentrations of each unknown solution were assayed and a standard curve (0.01 - 0.05  $\mu$ moles) 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<sub>3</sub>PO<sub>4</sub> 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 m $\mu$  against a reagent blank. A standard

the first time in the history of the world, the whole of the human race has been gathered together in one place, and that is the city of New York.

The second reason is that the United States is the most powerful nation in the world. It has the largest army and navy, and it is the only country that can afford to support such a large number of people.

The third reason is that the United States is a democratic country. It is based on the principle of "one man, one vote," which means that every person's voice counts.

The fourth reason is that the United States is a free country. It does not have a king or a queen, and it is not controlled by any one person or group.

The fifth reason is that the United States is a rich country. It has a lot of natural resources, and it has developed a strong industrial base.

The sixth reason is that the United States is a safe country. It has a well-trained police force, and it has a strong military defense system.

The seventh reason is that the United States is a peaceful country. It does not go to war unless it is absolutely necessary, and it tries to resolve conflicts through negotiation and diplomacy.

The eighth reason is that the United States is a progressive country. It is always looking for ways to improve its society and its government, and it is open to new ideas and new ways of thinking.

The ninth reason is that the United States is a diverse country. It has people from all over the world living there, and it尊重s and celebrates their differences.

The tenth reason is that the United States is a great country. It has a long history, a rich culture, and a bright future.

(31)

curve (0.02-0.2  $\mu$ moles) was constructed and 3 different concentrations of each unknown solution were assayed.

Succinic semialdehyde was assayed according to the procedure described by Waelisch (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 m $\mu$  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  $\mu$ moles/ml) of the authentic compound in borate buffer.



ResultsI. Incorporation of C<sup>14</sup>-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  $\mu$ moles 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<sup>14</sup>-glutamic acid into the collagen-containing hot TCA-extractable protein fraction. The amount of incorporation, however, was small. The spec-

and the  $\mathcal{L}$ -operator is defined by

$$\mathcal{L} = \frac{1}{2} \partial_x^2 + \frac{1}{2} \partial_y^2 - \frac{\alpha}{x^2} - \frac{\beta}{y^2} + \frac{\gamma}{x^2+y^2}$$

where  $\alpha, \beta, \gamma$  are real constants. The Schrödinger equation is

$$-\frac{\hbar^2}{2m} \nabla^2 \psi + V(x, y) \psi = E \psi$$

with potential

$$V(x, y) = \frac{\alpha}{x^2} + \frac{\beta}{y^2} + \frac{\gamma}{x^2+y^2}$$

The radial part of the wave function is given by

$$\psi(r) = r^{-1/2} R(r)$$

where  $r = \sqrt{x^2 + y^2}$ . Substituting this into the Schrödinger equation, we get the radial Schrödinger equation:

$$-\frac{\hbar^2}{2m} \left( \frac{1}{r^2} \frac{d^2 R}{dr^2} + \frac{1}{r} \frac{dR}{dr} \right) + V(r) R = E R$$

with potential

$$V(r) = \frac{\alpha}{r^2} + \frac{\beta}{r^2} + \frac{\gamma}{r^2} = \frac{\alpha + \beta + \gamma}{r^2}$$

Let's consider the case where  $\alpha = \beta = 0$ . Then the potential is  $V(r) = \frac{\gamma}{r^2}$ . The radial Schrödinger equation becomes

$$-\frac{\hbar^2}{2m} \left( \frac{1}{r^2} \frac{d^2 R}{dr^2} + \frac{1}{r} \frac{dR}{dr} \right) + \frac{\gamma}{r^2} R = E R$$

or

$$\frac{d^2 R}{dr^2} + \frac{1}{r} \frac{dR}{dr} + \frac{2mE}{\hbar^2} r^2 - \frac{\gamma}{r^2} R = 0$$

Table II

Incorporation of  $\text{C}^{14}$ -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  $\mu\text{moles}/\text{ml}$ ; 0.12 ml magnesium acetate 0.3 M; 0.63 ml KCl, 1.0 M; 0.12 ml GTP, 2.5  $\mu\text{moles}/\text{ml}$ ; 0.24 ml glutathione, 200  $\mu\text{moles}/\text{ml}$ ; 0.33 ml sodium phosphoenolpyruvate, 100  $\mu\text{moles}/\text{ml}$ ; 0.12 ml pyruvate kinase, 1 mg/ml; 0.12 ml ascorbic acid, 50  $\mu\text{moles}/\text{ml}$ ; 0.15 ml cysteine, 2  $\mu\text{moles}/\text{ml}$ ; 0.30 ml amino acid mixture; containing-L-proline, arginine, lysine, threonine, serine, leucine, phenylalanine, methionine, valine, asparagine, aspartic acid, isoleucine, histidine, tryptophan, alanine, tyrosine, and glycine at a concentration of 1.0  $\mu\text{mole}/\text{ml}$ . 0.50 ml of  $\text{C}^{14}$ -glutamic acid ( $28\text{uc}/\mu\text{mole}$ ,  $2.24 \times 10^6 \text{ cpm}/\text{ml}$ ) was added to vessels 1 and 2 before incubation and to vessel 3 after precipitation of the protein.

The incubation was carried out at  $38^\circ \text{C}$ . for 90 minutes.

<u>Vessel #</u>	<u>Contents</u>			<u>Total activity of collagenous protein</u>	<u>Specific activity of non-collagenous protein</u>
	pH 5	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

the first time in the history of the world, the  
whole of the human race has been gathered  
together in one place.

It is a remarkable fact that the whole of  
the human race has been gathered together  
in one place, and that the whole of the  
human race has been gathered together  
in one place.

It is a remarkable fact that the whole of  
the human race has been gathered together  
in one place, and that the whole of the  
human race has been gathered together  
in one place.

It is a remarkable fact that the whole of  
the human race has been gathered together  
in one place, and that the whole of the  
human race has been gathered together  
in one place.

It is a remarkable fact that the whole of  
the human race has been gathered together  
in one place, and that the whole of the  
human race has been gathered together  
in one place.

Table III

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

<u>Fraction</u>	<u>Macromolecular activity (total cpm)</u>	<u>Small molecular activity (total cpm)</u>
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  $\text{C}^{14}$ -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  $125^{\circ}\text{ C.}$  for

the first time in the history of the world, the whole of the  
population of the earth has been gathered together in one place,  
and that is the city of New York.

The reason why this is so is that there is no other place in the  
world where so many different nationalities are gathered together  
as in New York. There are people from every country in the  
world here, and they all live together in peace and harmony.  
This is a great blessing to the world, because it shows that  
people of different nationalities can live together in peace and  
harmony, and that they can work together for the betterment  
of the world.

Another reason why this is so is that New York is a  
great commercial center. It is the largest port in the world,  
and it handles more shipping than any other port. This  
means that there is a great deal of business going on in  
New York, and that there are many opportunities for  
people to earn a living here.

Finally, another reason why this is so is that New York  
is a great cultural center. It has many great museums,  
libraries, and theaters, and it is a center for art and  
culture. This means that there are many opportunities for  
people to enjoy themselves here, and to learn about  
the world and its people.

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 unarranged 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  $\mu$ moles 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 unarranged 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  $\mu$ moles recovered)

<u>Compound</u>	<u>Rearranged values adjusted to hydroxyproline recovered from</u>			<u>Difference</u>
	<u>Rearranged</u>	<u>Control</u>	<u>Control</u>	
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 and the succinic semialdehyde-2,4-dinitrophenyl-hydrazone 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 unarranged 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 unarranged 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.



III. The application of the Lossen rearrangement to the hot TCA-extractable protein derived from 14 day old chick embryos after incubation with C<sup>14</sup>-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<sup>14</sup>-glutamic acid ( $3.8 \times 10^6$  cpm/ml, 205  $\mu$ curies/ $\mu$ mole) and 0.9 ml of a solution of C<sup>14</sup>-glutamic acid ( $3 \times 10^6$  cpm/ml, 170  $\mu$ curies/ $\mu$ mole), the total activity being equivalent to 11  $\mu$ curies. 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 occasionally 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.

<u>Compound</u>	<u>Total yield</u> <u>(umoles)</u>	<u>Total activity</u> <u>(cpm)</u>
Glutamic acid	not assayed	195
Hydroxyproline	138.5	not counted
alpha, gamma-diamino butyric acid	not assayed	109
Succinic semialdehyde	3.3	0

1968.08.14

Black-capped N.

1968.08.14

Black-capped N.

The hydrolysate was filtered and the 2,4-dinitrophenyl-hydrazone 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,<sup>gamma</sup>-diaminobutyric acid and that none existed in the succinic semialdehyde. If the counts in alpha,<sup>gamma</sup>-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<sup>14</sup>-glutamate into alpha-linkage and its incorporation into gamma-linkage, such that a measurable 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<sup>14</sup>-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.

THE INFLUENCE OF THE CULTURE MEDIUM ON THE GROWTH OF *CHLAMYDIA*

BY J. R. HARRIS AND J. A. WILSON, JR., DEPARTMENT OF MEDICAL MICROBIOLOGY, UNIVERSITY OF TORONTO, TORONTO, CANADA

(Received January 19, 1966; accepted April 1, 1966)

ABSTRACT: The influence of various culture media on the growth of *Chlamydia trachomatis* was studied.

The results indicate that the growth of *C. trachomatis* is dependent on the presence of a living host cell. The growth of *C. trachomatis* is inhibited by the presence of antibiotics, particularly tetracycline.

The growth of *C. trachomatis* is inhibited by the presence of antibodies, particularly IgM antibodies.

The growth of *C. trachomatis* is inhibited by the presence of complement, particularly IgM complement.

The growth of *C. trachomatis* is inhibited by the presence of IgG antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgA antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgD antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgE antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgF antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgG antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgA antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgD antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgE antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgF antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgG antibodies.

The growth of *C. trachomatis* is inhibited by the presence of IgA antibodies.

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 unarranged control. There were 463  $\mu$ moles 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

9. In the same manner as in the previous section, we can show that

$$\lim_{n \rightarrow \infty} \int_X |f_n|^p d\mu = \int_X |f|^p d\mu.$$

Since  $\int_X |f|^p d\mu < \infty$ , we have

$$\lim_{n \rightarrow \infty} \int_X |f_n|^p d\mu = \int_X |f|^p d\mu < \infty.$$

Therefore,  $f_n \rightarrow f$  in  $L^p(X, \mu)$ .

10. By the same argument as in the previous section, we can show that

$$\lim_{n \rightarrow \infty} \int_X |f_n|^q d\mu = \int_X |f|^q d\mu.$$

Since  $\int_X |f|^q d\mu < \infty$ , we have

$$\lim_{n \rightarrow \infty} \int_X |f_n|^q d\mu = \int_X |f|^q d\mu < \infty.$$

Therefore,  $f_n \rightarrow f$  in  $L^q(X, \mu)$ .

11. By the same argument as in the previous section, we can show that

$$\lim_{n \rightarrow \infty} \int_X |f_n|^r d\mu = \int_X |f|^r d\mu.$$

Since  $\int_X |f|^r d\mu < \infty$ , we have

$$\lim_{n \rightarrow \infty} \int_X |f_n|^r d\mu = \int_X |f|^r d\mu < \infty.$$

Therefore,  $f_n \rightarrow f$  in  $L^r(X, \mu)$ .

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 $\mu$ moles recovered						Difference*	
	Control Gelatin			Rearranged Gelatin				
	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	+12.21	
$\mu$ moles Hydro- $\mu$ moles Succinic semialdehyde	-	-	-	18.7	19.7	19.2	-	

\* Difference between control average and rearranged average.

\*\* Duplicates = Duplicate elutions from duplicate electro-  
phoreses.

\*\*\* 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  $\mu$ moles of glutamic acid rearrangement products still leaves 118  $\mu$ moles unaccounted for out of the 139  $\mu$ moles 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 hydroxy-proline 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 of the 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 aminoacyladenylylate-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



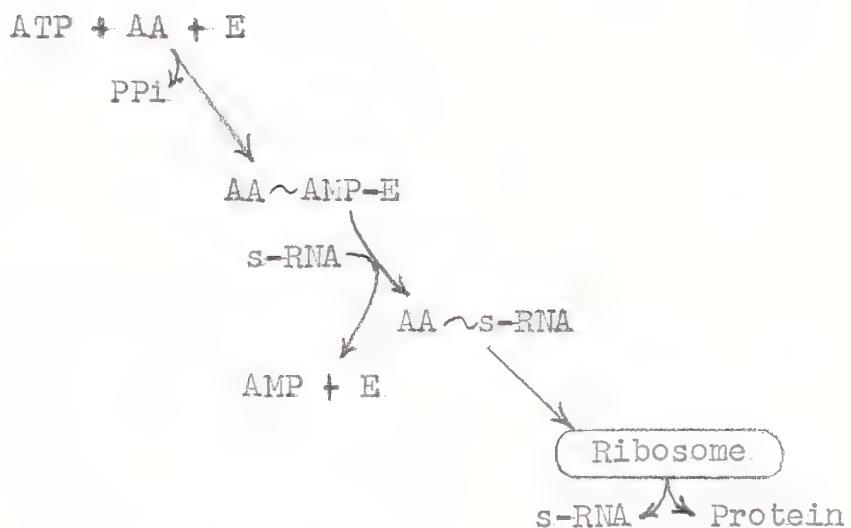


Fig. 5. Diagram of Protein Synthesis. E = enzyme,  
 AA = amino acid, PPi = inorganic pyrophosphate, s-RNA =  
 transfer RNA.



(52)

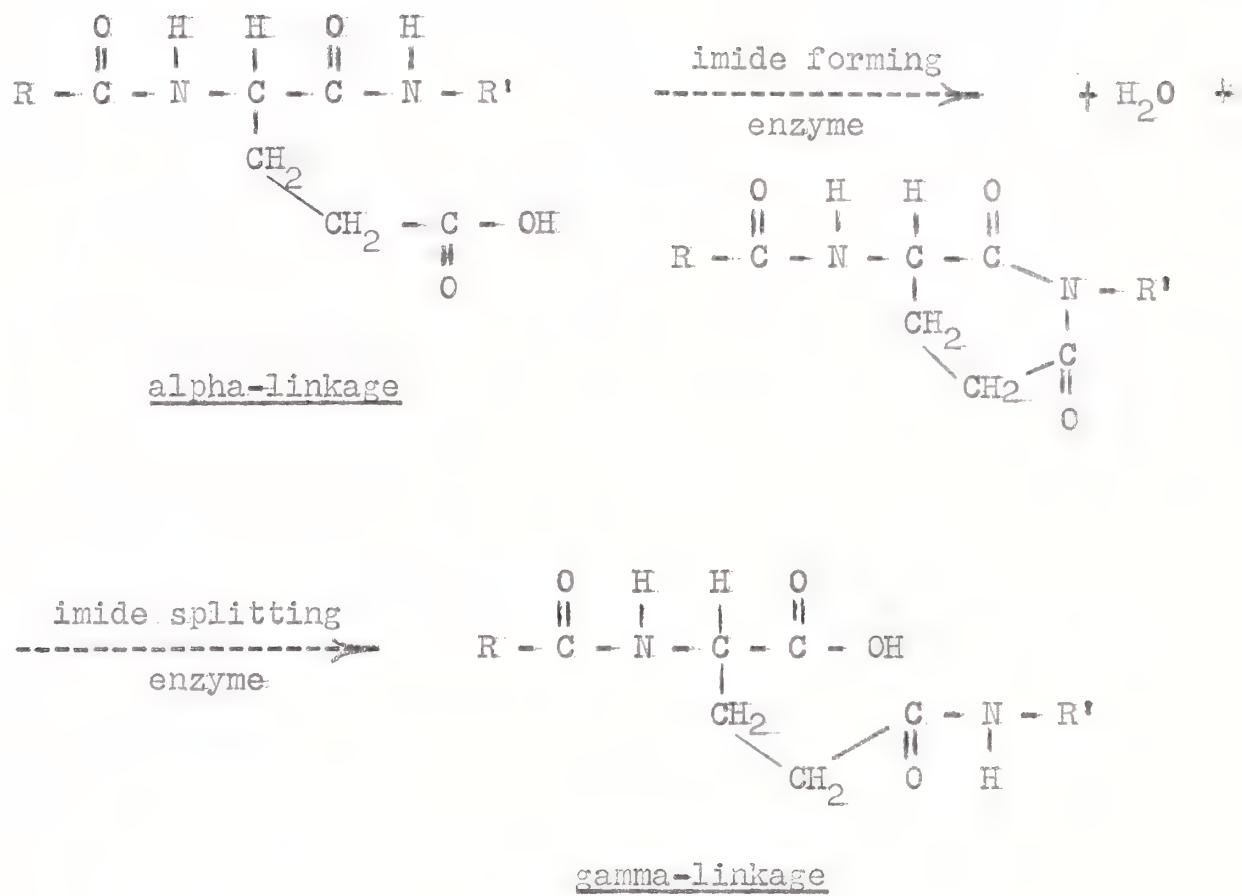


Fig. 6. Possible mechanism for intramolecular formation of gamma-glutamyl bonds.



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



(55)

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 unarranged 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 un-rearranged control. This collagen appears to contain at least 19  $\mu$ moles 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 1<sup>4</sup>-day embryos. After formation of the polyhydroxamate derivative, one half of the solution was rearranged and the other was used as an unarranged 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.

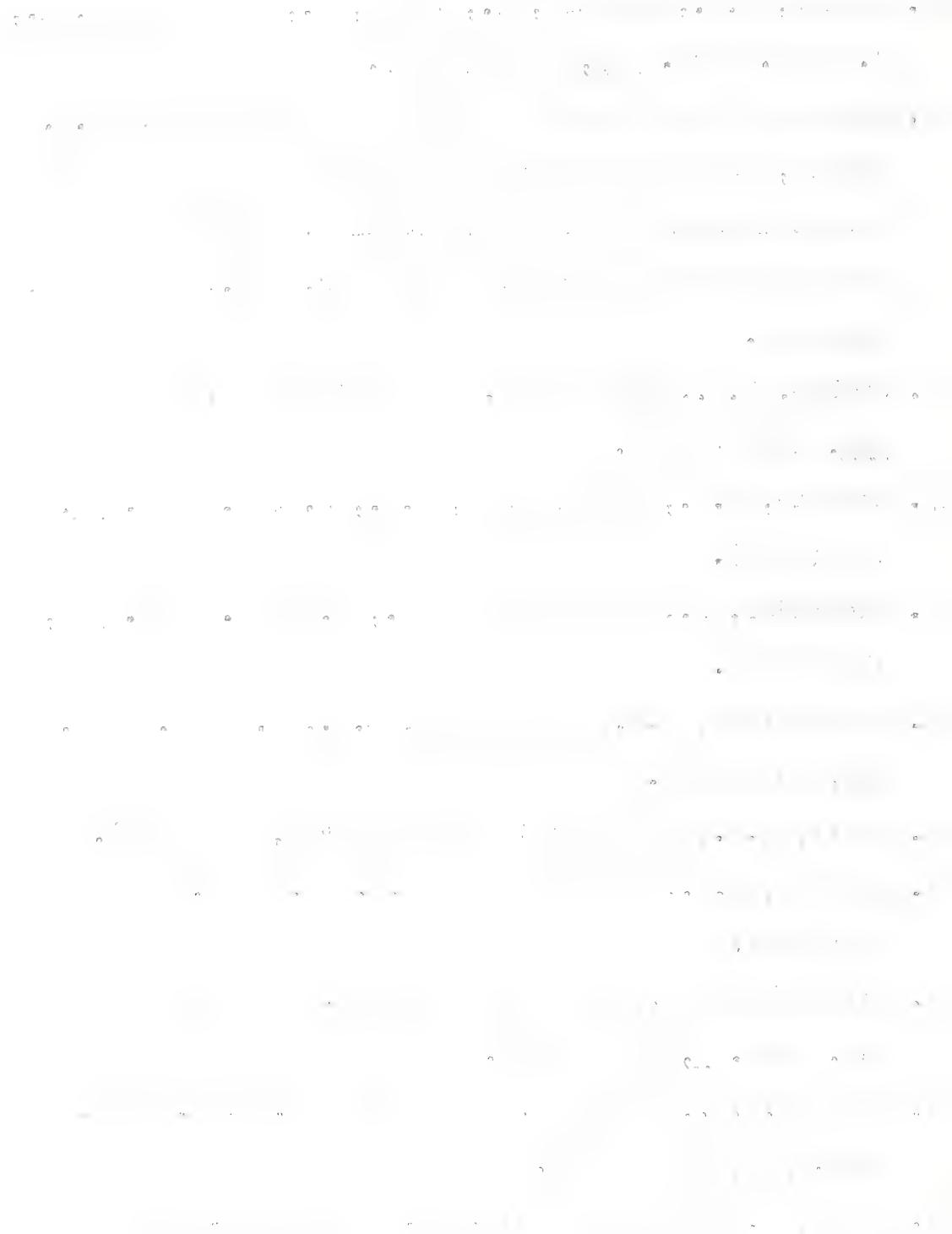


Bibliography

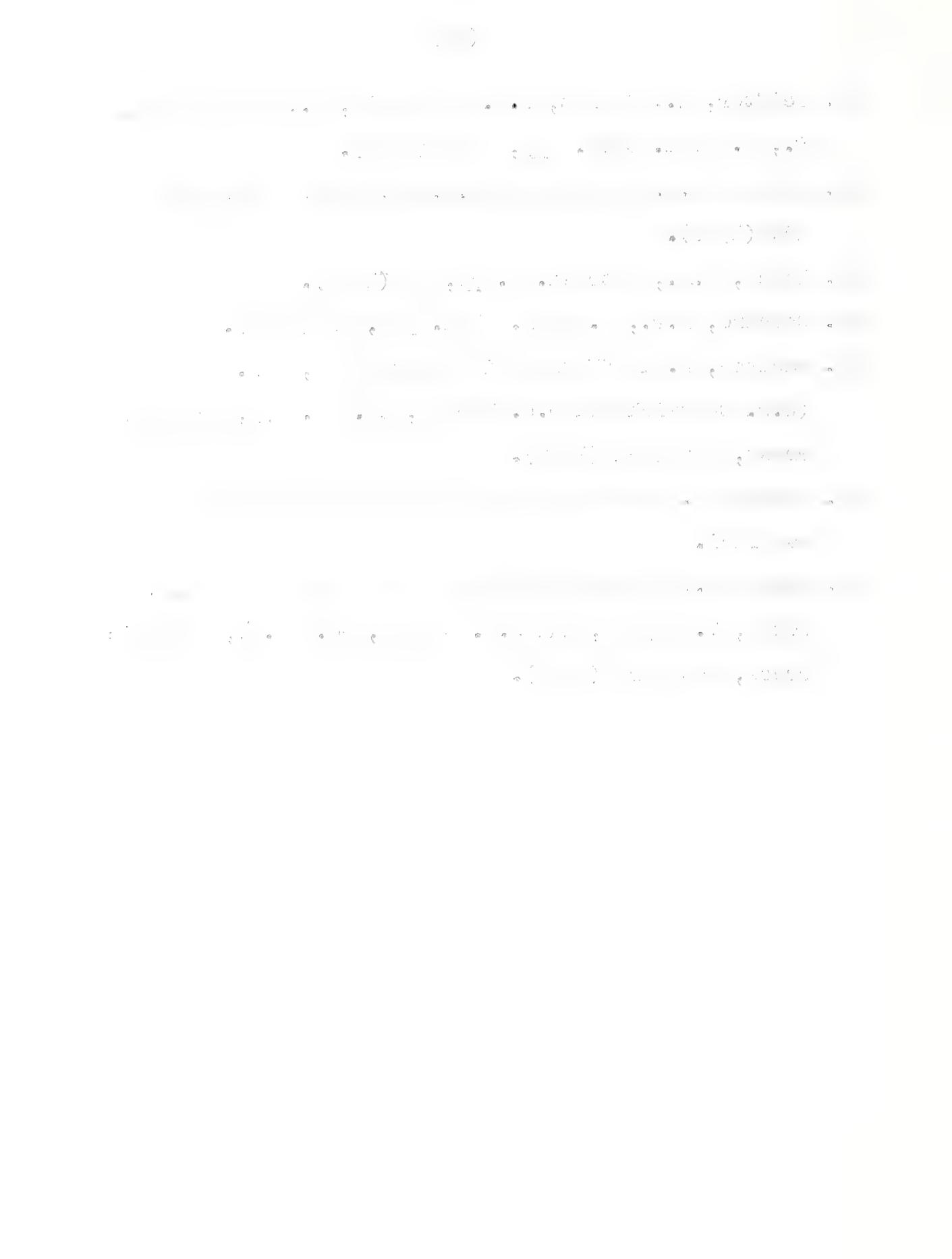
1. Quastel, J.H., Stewart, C.P., and Tunnicliffe, H.E., Biochem. J. 17, 586 (1923).
2. Dakin, H.D., J. Biol. Chem. 1, 171 (1905).
3. Hopkins, F.G., Biochem. J. 15, 286 (1921).
4. Kendall, E.C., Mason, H.L., and McKenzie, B.F., J. Biol. Chem. 87, 55 (1930).
5. Harrington, C.R., and Mead, T.H., Biochem. J. 29, 1602 (1935).
6. Ivanovics, G., and Bruckner, V., Zeitschrift für Immunitätsforschung 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., 1451 (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.H., 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. Waelisch, 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).







YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by \_\_\_\_\_ has been  
used by the following persons, whose signatures attest their acceptance of the  
above restrictions.

---

---

NAME AND ADDRESS

DATE

