UNIVERSITY OF CINCINNATI

June 3 1938

I hereby recommend that the thesis prepared under my supervision by J. F. Kowalewski

entitled a Micro Biological Study of Skin -

be accepted as fulfilling this part of the requirements for the degree of Ph. D.

Approved by: Geo. D. Laherty

FORM 668—G.S. AND ED.—1M—7-33

A Microbiological Study of Elastin and Collagen

A Thesis

by

J. F. Kowalewski

Presented to the Faculty

of the

University of Cincinnati

in fulfillment of part of the requirements for the degree of

Doctor of Philosophy

Cincinnati, Ohio

Degrees:

B. S. University of Chicago

M. S. University of Cincinnati

ACKNOWLEDGEMENT

The writer wishes to make grateful acknowledgement to Dr. Fred O'Flaherty for his valuable help, guidance, and encouragement throughout this investigation.

A Microbiological Study of Elastin and Collagen

A. Introduction and History.

1. Purpose:

The action of micro-organisms on skin as a whole has been studied considerably, both from the theoretical and practical view-points, but the study of micro-organisms upon specific constituents of the skin has received little attention. In this investigation, a study was made of the action of micro-organisms upon collogen and elastin.

This study included the following:

a. relative rates of attack

b. factors affecting bacterial action

c. study of the hydrolytic products in the light of the polypeptide linkage.

A. Introduction and Historical Discussion.

II. Historical Discussion.

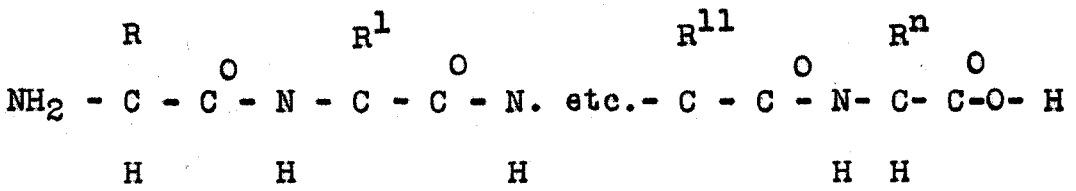
a. Proteins.

A theoretical study of proteins began perhaps, in 1830, when Mulder worked on albumin in an effort to determine an empirical formula for proteins. This was followed by contributions from Mussner ( ), Kossel (14), Kuhne (15), Fischer (9), Abderhalden (1), Brill (5), Troingsord (21), Korrer (13), Ssadikow (20), Astbury (3), Bergmann (3), Highberger (12).

Before discussing several of the recent theories regarding proteins and enzymes, it might be well to summarize the trend of investigation and thought pursued by those who contributed to protein and enzyme chemistry. The early investigators, because of their limited knowledge, were often forced to build their theories on meager experimental foundations and later obtained the confirmation of their assumptions from additional experimental facts. This form of deductive reasoning was characteristic of the organic chemist, and it was the organic chemist who first attacked the problems of proteins and enzyme chemistry. The chemist, then, always

attempted to obtain an analysis of the compound under investigation, and later, with all the information available, he tried to build up the same original compound. The synthesis was the confirmation of the analysis, and consequently the solution of the problem. The proteins apparently were one of the first major obstacles in the path of the organic chemist's scheme of investigations. The early investigator soon realized that the older methods were entirely too limited to aid him in elucidating the detailed complexity, so efforts were made to improve laboratory technique and introduce newer methods. At this point, enzymes chemistry was brought into use. The action of enzymes, always was, and still is, regarded as the most sensitive criterion in the study of the decomposition products of proteins. With newer facilities, the scientist was able to further his speculations, formulate new hypotheses, and finally have these hypotheses develop into theories. Later, one observes that the newer theories begin to take on a physical nature, and today, we find both physics and mathematics assisting the chemist in his investigations in protein and enzymes chemistry. Recent theories regarding the structure of the protein molecule will be briefly discussed, so that a rela-

tionship may be seen between the data presented in in this thesis and the present day knowledge of proteins. The modern view of the protein molecule has been given to us by Bergmann and Astbery. Bergmann, whose work has been primarily chemical, suggests the following structure:



A fiber-like protein (collagen, silk fibrin, etc.) contains a series of the above linkages lying parallel to each other, definite distances apart. The C O O H group of one amino-acid linked with one NH_2 group of another amino-acid, with the elimination of water. The R, R^1, R^{11}, and R^n, being side chains. They may be aliphatic, aromatic, or heterocyclic groups: they may contain OH or S; or they may be neutral, acidic or basic. Bergmann thus concludes that the natural proteins are not simply polypeptide chains of every conceivable length and configuration, but that their structures are defined by a general stoichiometrical law which greatly limits the number of possible formulas, and that every individual amino-acid residue in the peptide chain of the proteins molecule recurrs

at constant intervals. From Bergmann's and Astbury's studies, proteins contain 288 amino-acid residues, or whole multiples of 288. Bergmann was able to work out tables showing the frequencies of various amino-acids of cattle fibrin, silk fibroin, and other proteins. The tables were based upon conclusions drawn from chemical analyses of proteins. It might be mentioned at this point, that the present day theories of proteins are based upon Fischer's most valuable work done in the early part of this century.

Astbury studied proteins by means of X-Rays. He observed two kinds of proteins, the non-fibrous (crystalline) and the fibrous (non-crystalline). To the crystalline type belong such proteins as egg albumin, haemoglobin, and insulin, while to the fibrous proteins, there are silk, hair, muscular and connective tissues.

The natural fibrous proteins show an X-Ray pattern which, when interpreted, reveals a structure similar to that sponsored by Bergmann. Astbury studied stretched and unstretched keratin, and found that two forms of keratin exist. The original unstretched molecular form, he called "Alpha-keratin", and the stretched form he called "Beta-keratin".

X-Ray study of globular proteins led Astbury to postulate a globular molecule similar in principle to the fibrous molecule, but constituting multiple folds or coils of polypeptide chains.

Later X-Ray studies on dentaured globular proteins revealed that this changed molecule took on the appearance of a polypeptide chain similar to fibrous protein.

Generalizations drawn from Astbury's work would lead one to think of proteins as structures of Bergmann's polypeptides differing in configurations and multiplicity of folds. First the short molecule (globular protein), then the less contracted state (fibrous protein), lastly the fully-extended configuration, the Beta, or stretched form.

A. Introduction and Historical Discussion.

II. Historical Discussion.

b. Enzymes.

Likewise, a definite advance in the study of enzymes began with Kirchoff ( ) in 1815. Other contributors to this work were ~~Gay-Lussac~~ Falk (8), Eberle (7), Schwann (18), Corvisant (9), Pasteur ( ), Kuhne (15), Buchner (6), Harden (11), ~~Young~~ Vickery (23), Sorensen (19), Michaelig (16), Willstatter (22), Fodor (10), and Bergmann (4).

Since this work dealt with micro-organisms, and they in turn perpetuate their existence by utilizing nourishment rendered by assimable actions, it appears important at this point to briefly discuss present day notions regarding enzymes.

Most workers in the field of enzymes today believe an enzyme to be colloid-like in nature, essentially a protein, possessing two components, a carrier, of polypeptide structure, and an active substance, the former having a varying degree of power to enhance or depress the activity of the latter. The nature of the substrate likewise is an important factor in enzymatic reactions.

A review of the literature on enzymes will convince one that at the present time we know enzymes primarily by their actions.

B. Experimental.

I. Isolation of Micro-organisms.

The micro-organisms used in this work were obtained from soak waters (from tanneries), from the upper layer of soil, and from the effluent of a septic tank. By soak water is meant the water in which hides are soaked before unhairing. This appeared the most logical place to obtain the proper strains of bacteria for this work.

Bags of cheese-cloth containing elastin and collagen were suspended into soak waters for 48 hours, the tissues were then placed into sterile media and cultured. These cultured colonies were isolated. A total of 134 strains of aerobic and an-aerobic bacteria were isolated. Twenty-five strains of bacteria were obtained from the soil, and fifteen from the effluent of a septic tank. Pure cultures of organisms were first grown on nutrient agar and in broth. Twenty-four-hour sub-cultures of the aerobic strains were transferred to gelatine and incubated for four days at 27°C. This was done to identify the proteolytic bacteria. Thirty-one strains of proteolytic aerobes were obtained.

After eliminating the faculative anaerobes, cul-

tures of anaerobes were grown, and seven strains were obtained. Of the seven strains of anaerobes isolated, three were proteolytic.

B. Experimental.

II. Preparation of Tissue.

The material used in this experiment was biologically pure elastin and collagen. The elastic tissue was obtained from ligamentum nuchae, a ligament located in the neck of grazing animals. It is a known fact that the yellow elastic tissue behaves much like the elastin of the skin.

The collagen was obtained from the tendons of the extensor leg muscles of cattle. These tissues were placed in saline solution immediately after slaughter, and within three hours, all extraneous tissue was removed. It was then cut into small strips $\frac{1}{16} \times \frac{1}{16} \times 2"$ and washed repeatedly with physiological saline solution (0.9% NaCl) to remove albumins and globulins. The tissue was then permitted to soak in distilled water for twenty-four hours at 10°C.

The tissues were further prepared by two different methods. The first method consisted of drying the tissue over $CaCl_2$ as 10°C for several days, and then grinding it very fine in a Wiley mill.

In the second method, which more closely approached the conditions as existant in skin, the tissues were removed from the saline solution and cut in very thin layers by means of a freezing microtone.

B. Experimental.

III. Sterilization of Tissue.

One-half gram of the respective tissue was placed in test-tubes (150x18 mm.) containing 10 ml. of the medium. The tubes were stoppered with cotton and heated on a water bath at 65°C for one hour, then incubated for twenty-four hours at 27°C. This procedure of heating and incubating was repeated four times.

B. Experimental.

IV. Inoculum.

The pure cultures of the aerobes were grown for forty-eight hours; the anaerobes for ninety-six hours. The colonies were washed off in sterile physiological saline solution and diluted to an arbitrary standard of opacity which resulted in a concentration of approximately 500-600 million organisms per ml.

One ml. of this suspension was then diluted with 100 ml. of sterile physiological saline solution, and after thorough mixing, 1 ml. of this solution was used as the inoculum for each tube.

This method of dilution was adopted in order to reduce to a minimum the amount of medium or nitrogenous products which might be carried over in the washings.

B. Experimental.

V. Hydrolysis produced by Micro-organisms.

The test tubes containing the inoculated elastin and collagen were incubated for seventy-two hours at a temperature of 27°C. At the end of this period, the liquid from each individual test-tube was filtered from the solid material by means of a No. O Munktell filter paper and washed with a number of 5 ml. portions of distilled water until the volume of the filtrate increased to about 50 ml. To the filtrate from each test tube, 10 ml. of concentrated H_2SO_4 (sp 1.84) was added, and then sufficient distilled water to make the volume 100 ml. The nitrogen content of each filtrate was then determined by micro-kjeldahis method of Pregel.

Based upon the results of the experiment, strains 16, 104, 108, 110, and 205 were selected for further study.

The elastin and collagen tissues were subjected to aerobic strains for 3, 5, and 7 days, and for three weeks for the one strain of anaerobe.

Table I: Experimental.

The percent of Nitrogen contained in the Solubilized Portion of Elastin and Collagen after three days of Attack by Various Strains of Proteolytic Bacteria.

Strain No.	Character of Organisms	Percent Nitrogen dissolved*	
		Elastin	Collagen
-	Blank no inoculum	0.035	0.042
1	Gram negative slender pleomorphic bacillus	0.390	0.393
2	Gram positive thick-terminal spored bacillus	0.175	0.539
3	Gram negative long slender bacillus	0.276	0.739
4.	Gram negative pleomorphic bacillus	0.162	0.856
5	Gram negative short bacillus	0.573	0.749
6	Gram negative pleomorphic bacillus	1.340	1.599
7	Gram positive long rounded end bacillus	1.463	2.387
8	Gram positive small uneven staining bacillus	0.087	1.466
9.	Gram positive slender uneven staining bacillus	0.323	2.279
10	Gram negative pleomorphic bacillus	1.001	1.771
11	Gram positive thick pleomorphic bacillus	0.893	2.263
12	Gram positive thick pleomorphic bacillus	0.285	2.325
13	Gram positive long slender diphtheroid bacillus	0.585	2.340

Table I: (Cont.)

Strain No.	Character of Organisms	Percent Nitrogen dissolved*	
		Elastin	Collagen
14	Gram positive thick rounded end bacillus	1.125	1.275
15	Gram negative pleomorphic bacillus	1.800	2.595
16	Gram negative filamentous and branching	7.725	8.100
17	Gram negative pleomorphic bacillus	2.490	3.315
18	Gram positive staphylococcus	2.205	3.330
19	Gram negative filamentous (like 16)	0.120	1.485
20	Gram negative long slender bacillus	0.570	2.700
	The above 20 strains were obtained from soak water.		
103	Gram positive long slender spore forming bacillus	0.390	0.990
104	Gram negative short thick bacillus	0.555	3.960
105	Uneven staining pleomorphic bacillus	0.390	0.705
107	Gram positive thick spore forming bacillus	0.165	0.825
108	Gram negative very small ovoid bacillus	0.825	1.550
109	Gram negative long slender bacillus	0.375	1.125

Table I: (Cont.)

Strain No.	Character of Organisms	Percent Nitrogen dissolved*	
		Elastin	Collagen
110	Gram negative small pleomorphic bacillus	0.675	5.445
111	Same appearance as 105	0.135	0.345
	Strains 103 to 111 inclusive obtained from soaked hide.		
201	Gram negative long slender bacillus		
202	Gram positive long slender bacillus		
203	Gram negative long slender bacillus	non-proteolytic	
204	Uneven staining long curved bacillus		
205	Gram positive spore forming bacillus	proteolytic	
206	Gram positive spore forming bacillus		
	Strains 201 to 206 obtaind from soaked hide.		

*Determined by micro-Kjeldahl and based upon the dry weight of the original samples.

Table II: Experimental.

The percent of Nitrogen contained in the Solubilized Portion (Filtrate) of the Elastin and Collagen Tissues after three, five, and seven days Attack by Bacteria.

Strain	Tissue	Percent of N_2 in filtrate as determined by Micro-Kjeldahl*)			Percent of N_2 in filtrate, determined by Micro van Slyke*)	Percent of Amino N_2 in filtrate**)
		3 days	5 days	7days		
16	Elastin	7.875	8.745	10.88	8.48	77.09
	Collagen	8.328	9.222	14.76	10.92	75.42
104	Elastin	0.620	3.000	--	--	--
	Collagen	4.120	5.775	--	--	--
108	Elastin	0.822	3.335	--	--	--
	Collagen	1.602	4.815	--	--	--
110	Elastin	0.625	0.975	1.20	10.60	89.01
	Collagen	5.540	7.395	8.87	7.34	82.27
Control no organism	Elastin	0.025	0.560	0.06	--	--
	Collagen	0.042	0.068	0.07	--	--
205	Elastin	after 3 wks.		10.11	8.69	86.70
	Collagen			15.03	12.15	80.70

*) Based upon the dry weight of the samples.

**) Based upon Kjeldahl nitrogen as 100.

The work thus far showed that collagen and elastin were not attacked alike. The analysis showed a greater percent of total nitrogen in the collagen filtrate than in the elastin in each case. Also that the percen of N_2 as amino nitrogen is higher for elastin than for collagen in each case.

B. Experimental.

VI. Effect of ions upon Bacterial Action.

I next studied the effect of various ions upon the rate of hydrolysis of elastin and collagen produced by the various organisms.

The following table contains the ions used and experimental data:

Table III

$ZnCl_2$	X	$CaCl_2$	X
$ZnSO_4$	X	$CaSO_4$	X
$Zn(NO_3)_2$	+	$Ca(NO_3)_2$	+
$MnCl_2$	+	$CnCl_2$	−
$MnSO_4$	+	$CnSO_4$	−
$Mn(NO_3)_2$	++	$Cn(NO_3)_2$	−
$FeCl_3$	X	NH_4Cl	++
$Fe_2(SO_4)_3$	X	$(NH_4)_2SO_4$	++
$Fe(NO_3)_2$	X	$(NH_4)NO_3$	+++

X--means no effect upon hydrolysis

+--increase in speed of hydrolysis

−--decrease in speed of hydrolysis

From the data in Table III it may be concluded that NH_4NO_3 promotes hydrolysis best. This salt was then used in the medium during the remainder of the work.

B. Experimental

VII. Optimum pH.

Collagen and elastin were next subjected to the various organisms at a pH of 5.5, 6.5, 7.5 and 9 for the purpose of determining the optimum pH for these organisms.

Table IV--Effect of pH upon proteolytic bacteria

Organism	pH 5.5	6.5	7.5	9
16	no growth	no growth	optimum	very slight
104	no growth	no growth	optimum	very slight
108	no growth	no growth	optimum	very slight
205	no growth	no growth	optimum	very slight

The results were the same in the instance of collagen and elastin.

From this point on 1 X 10^{-4} motor NH_4NO_3 was added to the medium and the pH adjusted to 7.5.

B. Experimental

VIII. Quantitative determination of Basic-amino acids and Mono-amino acids.

Twenty test tubes, each containing .5 gms. of elastin, and twenty other test tubes, each containing .5 gms. of collagen (10 mls. of medium), were inoculated with strains 16; the entire procedure was repeated with strains 104, 108 and 205. The bacterial action continued for three days, the contents of the tubes were then filtered and to the filtrate a disinfectant was added to prevent further organism activity. The filtrate was then diluted to a definite volume after whish Micro-Kjeldahls and van Slykes were run.

Table V--Per cents of N_2, Basic and Mono-amino acids of Elastin

Organism	Time	Total N_2 by Micro-Kjeldahl	Per cent of Basic amino acid	Per cent of Mono-amino acid	Per cent of NH_3
#4	3 days	65.54%	7.85%*	86.79%	.32%
#6	3 days	67.92%	7.10%	86.42%	.19%
#8	3 days	62.35%	6.72%	85.62%	.31%
#1 (anaerobe)	3 days	68.65%	7.93%	84.89%	.18%

*The total nitrogen in the filtrate was considered as 100% when calculating the per cent of basic amino acids, mono-amino acids and free ammonia.

Table VI--Per cents of N_2, Basic and Mono-amino acids of Collagen

Organism	Time	Total N_2 by Micro-Kjeldahl	Per cent of Basic amino acid	Per cent of Mono-amino acid	Per cent of NH_3
#4	3 days	79.51%	19.6%*	74.97%	.9%
#6	3 days	80.15%	21.02%	72.95%	1.4%
#8	3 days	74.62%	19.8%	73.92%	.96%
#1 (anaerobe)	3 days	81.65%	20.95%	71.85%	.65%

*The total nitrogen in the filtrate was considered as 100% when calculating the per cent of basic amino acids, mono-amino acids and free ammonia.

B. Experimental.

IX. Determination of Basic Amino-acids and Mono-Amino-acids formed by the micro-organism during definite intervals of time.

Thirty test tubes containing 10 mls. of medium and .5 gms. of elastin and thirty test tubes containing 10 mls. of medium and .5 gms. of collagen were inoculated with organism #6.

At the end of 36 hours activity by the micro-organism five tubes of each tissue were filtered and the filtrate was used in making micro-Kjeldahl and micro-van Slyke determinations. At intervals of each twelve succeeding hours this was repeated.

Table VII--Elastin—organism #6.

Time	% N_2 in Filtrate	Basic amino acid	Mono-amino acid	NH_3
36 hours	11.4%(1)	8.5%(2)	84.85%	.2%
48 hours	16.95%	8.92%	84.12%	.19%
60 hours	32%	7.35%	87.69%	.25%
72 hours	68%	7.31%	87.92%	.21%
84 hours	84%	7.48%	86.46%	.32%

(1) The total N_2 in elastin is 16.7% and the nitrogen in this column indicates the per cents of nitrogen considering the 16.7% to be 100%.

(2) The total nitrogen in the filtrate was considered as 100% when calculating the per cent of basic amino acids, mono-amino acids and free ammonia.

Table VIII--Collagen-organism #6.

Time	% of N_2 in Filtrate	Basic amino acid	Mono-amino acid	NH_3
36 hours	12.3%(1)	18.28%(2)	73.8%	.6%
48 hours	18.42%	18.62%	73.2%	.8%
60 hours	38.64%	18.33%	74.34%	.82%
72 hours	81.35%	21.82%	73.78%	1.2%
84 hours	82.78%	21.25%	72.81%	2.2%

(1) The total N_2 in elastin is 16.7% and the nitrogen in this column indicates the per cents of nitrogen considering the 16.7% to be 100%.

(2) The total nitrogen in the filtrate was considered as 100% when calculating the per cent of basic amino acids, mono-amino acids and free ammonia.

In determining the basic amino acids and mono-amino acids the standard van Slyke method was used. Since the van Slyke and Micro-Kjeldahl procedures are found in any standard book on protein chemistry it is not included.

C. Discussion of Results.

Since micro-organisms do not attack collagen and elastin alike it is apparent that elastin and collagen differ physically or chemically. Likewise the hydrolytic products are quite different in each case.

The much higher per cent of basic amino acids found in this investigation, especially in the case of elastin, can be easily explained. In acid or basic hydrolysis of elastin only 60% of the total nitrogen has been accounted for in terms of amino acids and in this investigation 84% of the total nitrogen present in elastin has been found to be present in the amino acids resulting from the hydrolysis of elastin by micro-organisms. Apparently the basic amino acids had not been included in products produced by acid hydrolysis of elastin.

A study of the ions tested in this investigation and a knowledge of bacterial food demand makes it quite logical to suspect that NH_4NO_3 should prove to be the most beneficial for accelerating bacterial hydrolysis, since it contains nitrogen in its most oxidized and reduced state.

In observing closely the data on Tables V, VI, VII and VIII one will notice the close similarity in the per cents of basic amino acids and mono-amino acids produced by the hydrolytic action of the various proteolytic

organisms. No doubt a more true relation exists between the amino acids produced from proteins by micro-organisms and the molecular structure of the protein than by the more harsh methods, namely, acid and basic hydrolysis.

The fact that the per cent of basic amino acids produced by micro-organisms upon elastin decreases with time and with collagen the basic amino acids increase with time can be explained only by further investigation. Perhaps here might lie a bit of knowledge which might be of value in prying open the mystery of the protein molecule.

D. Summary:

I. Micro-organisms do not attak elastin and collagen alike.

a. All micro-organisms which attack elastin attack collagen at the same or greater rate.

b. Some organisms will attack collagen but not elastin.

c. From a study of a large number of organisms none was found to attack just elastin and not collagen.

II. The per cent of amino nitrogen was always found to be greater in elastin than collagen.

III. The $NH_4 NO_3$ was found to be most beneficial in increasing the rate of hydrolysis.

IV. The per cents of basic amino acids both in elastin and collagen were found to be far greater than in acid or basic hydrolysis.

V. The per cent of basic amino acids produced by micro-organisms or elastin decreased slightly with increase in quantity of the tissue.

VI. The per cent of basic amino acids produced by micro-organisms on collagen increase with an

increase of quantity of the tissue.

VII. The fact that results are somewhat different in this investigation from results obtained by acid and basic hydrolysis of elastin and collagen reveals the need of further research to arrive at some possible explanation for this difference and an explanation for other peculiarities observed in this work.

E. Bibliography.

1. Abderhalden, E., "Untersuchungen über Aminosäure, Polypeptide und Proteine" I & II, (1899-1919).
2. Astbury, W. T., "Fundamentals of Fibre Structure", Oxford University Press, 1933; Trans. Faraday Soc., 29, 193 (1933).
3. Bergmann, M., "Über neuere Proteinchemie", Naturwissenschaften, 12, 1155 (1924). Also J. Biol. Chem., 115, 77 (1936).
4. Bergmann, M., "Newer Biological Aspects of Protein Chemistry", Sci., 86, 187 (1937).
5. Brill, R., "Über Seidenfibroin", I., Ann., 434:204-217 (1923).
6. Buchner, E., "Über fraktionierte Fällung des Hefepressaftes", Biochem. Z., 9, 436 (1908).
7. Eberle, N., "Über das Wesen des Verdauungsprozess", Archiv., 30, (1836).
8. Falk, K. G., "Studies on Enzyme Action", J. Am. Chem. Soc., 35, 217 (1915). J. Biol. Chem., 53, 75 (1922).
9. Fischer, E., "Untersuchungen über Aminosäure, Polypeptide und Proteine" I & II, (1899-1919).
10. Fordor, A., "Mechanism of Enzyme Action", Current Sci., 4, 879 (1936).
11. Harden, A., "The Alcoholic Ferment of Yeast Juice", Proc. Roy. Soc., 77, 405 (1906).

12. Higburger, J. H., "X-Ray Spectographic Studies of Tanning Processes", J. Am. Leather Chem. Assoc., 33, 16, (1938).

13. Kârrer, P., "Zur Kenntnis der Aminosäuren", Helv. Chem. Acta, 7, 763 (1927).

14. Kossel, A., "Über die bösischen Stoffe des Zellkems", Z. physiol. Chem., 22, 176 (1896).

15. Kuhne, W., "Über die Verdauung der Eiweissstoffe durch das Pankreassaft", Vinh. Archiv. path. Anat., 39, 130 (1867).

16. Michaelis, L., Biochem. Z., 7, 1907 to 105, 1920.

17. Pasteur, "Oeuvres De Pasteur", Tome 11, "Fermentations et Generations Dites Spontaneis"; Maison et Cie, Editeurs; Paris.

18. Schwann, T., "Über das Wesen des Verdauungsprozess", Archiv., 30, (1836).

19. Sorensen, S. P., "Enzymstudien", Biochem. Z., 7, 407, 1908; ibid. 22, 352 (1909).

20. Ssadikow, W. S., "Über Produkte der katalytischen Spaltung von Eiweissstoffen", Biochem. Z., 136, 241 (1923).

21. Troensigaard, N., "Über die Konstitution der Eiweissverbindungen", Z. angew. Chem., 38, 623, (1925).

22. Willstatter, R., Z. physiol. Chem., 110, 232 (1920); ibid., 42, 245 (1925).

23. Vickery, H. B., "A Review of Hypothesis of the Structure of Proteins", Physiol. Rev., 8, 393 (1928).