# UNIVERSITY OF CINCINNATI

May 28 19 53

*I hereby recommend that the thesis prepared under my supervision by* George M. Sleichter

*entitled* A Study of the Glyoxal-Collagen Reaction as it Contributes to Cross Linking

*be accepted as fulfilling this part of the requirements for the degree of* Doctor of Philosophy

*Approved by:*

Form 668—G.S. and T.C.—1M—4-51

# A STUDY OF THE GLYOXAL-COLLAGEN REACTION AS IT CONTRIBUTES TO CROSS LINKING

A dissertation submitted to the

GRADUATE SCHOOL OF ARTS AND SCIENCES

of the University of Cincinnati

in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

1953

by

George M. Sleichter

B. Sc. Dickinson College, 1932
M. Sc. University of Cincinnati, 1939

ACKNOWLEDGEMENTS

The author wishes to extend his sincere gratitude to Dr. Fred O'Flaherty, Director of the Tanners Council Laboratory, for making this work possible.

Grateful appreciation is extended to Dr. Robert M. Lollar under whose valued guidance this investigation was carried on and to Mr. W. T. Roddy for his generous and helpful counsel.

To Mr. Charles O Dhonau, President of the Cincinnati College of Embalming, appreciation is expressed for his cooperation and generous allotment of time for the execution of this investigation.

TABLE OF CONTENTS

# ABSTRACT

Much interest in recent years has centered upon the mechanism responsible for the chemical stabilization of fibrous protein structures by treatment with aldehydes and other tanning agents. Among the several mechanisms proposed, the theory of cross-linkage has received much attention and study. These investigations have led to great advances in the elucidation of the molecular organization of such materials and also to the development of such modifications of the original fibrous proteins as has resulted in the enhancement of their properties for textile manufacturing purposes. It was the purpose of this investigation to study the stabilization of collagen which resulted upon treatment with the bifunctional aldehyde glyoxal.

Various types of tension studies in both the wet and dry states have been the most fruitful methods for the qualitative and quantitative evaluation of interchain forces in protein fibers. This study, utilizing the process of wet tension testing, has produced some evidence to support the theory that:

a. The treatment of collagen with glyoxal does introduce new and stronger cross-links between the polypeptide chains.
b. The introduction of such cross-links increases the hydrothermal stability of collagen.
c. The introduction of such cross-links increases the ultimate strength of the fiber.

The technique involved the use of a mercury-loaded tester by

which the stress-strain characteristics of the collagen fibers can be measured. The investigation consisted of two phases:

a. The first phase comprised a study of the stress-strain properties of micro-fibers with emphasis upon the tenacity expressed in terms of the mean breaking length, i. e. that length of the fiber which, if suspended from one end, would break under the load of its own weight. The results show that the tensile strength and shrinkage temperature increases and then levels off at a maximum as the amount of glyoxal given increases.

b. The second phase consisted of stress-strain measurements of thermally contracted gross fibers. Upon denaturation by heat in the presence of water or other liquids or solutions, collagen fibers contract to approximately one-fourth their original length. The shrunken or dematured material is highly elastic when maintained in the swelling medium. It was desired to study the stress-strain properties of the denatured or rubbery form of collagen by applying the theory of rubber elasticity. From data obtained during the measurements, the molecular weight of the collagen between cross-links can be calculated. The results show a drastic decrease in molecular weight and then a leveling off at a minimum as the amount of glyoxal given increases. Further, the reaction with glyoxal increased the modulus of elasticity of the shrunken collagen.

These data indicate that cross-linking plays a significant role in the action of glyoxal on collagen.

# INTRODUCTION

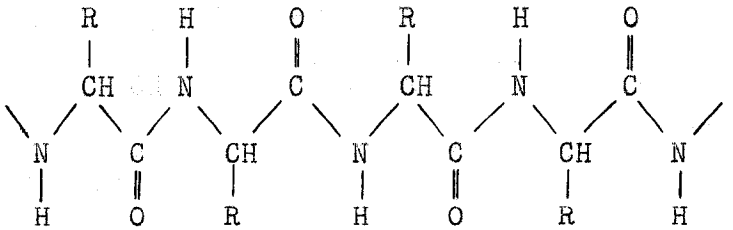
During the past decade many investigations designed to correlate the structure of fibrous materials with their mechanical properties have been completed. By far the most fertile field for researches of this character has been in the category of those fibrous materials whose properties and availability render them useful for the production of textile materials such as wool, silk, nylon, rayon, and cellulose (8)(17)(9).

As a result of such investigations great advances have been made in the elucidation of the molecular organization of such materials. These studies have also led to the development of such modifications of the original materials as has resulted in the enhancement of their properties for textile manufacturing purposes (4).

It is the purpose of this dissertation to report an investigation of the stabilization of the fibrous protein collagen upon treatment with the bifunctional aldehyde glyoxal. Previous investigators (6)(7) have reported the use of glyoxal as a tanning agent but little is known about the mechanism of this reaction, or the forces which result in the chemical stabilization of the fibrous proteins so treated. A brief review of the molecular organization of several contrasting fibrous proteins is given in order to serve as background material for this presentation.

Some of the most fundamental studies on protein fibers have led to the conclusion that they consist, in general, of polycondensation

products of amino acids in which the structural units are linked together to form a polypeptide chain which in its most general form may be represented in the following way:



This particular polypeptide arrangement is believed to be common to all the proteins including those of a fibrous nature such as wool, silk, and collagen. In spite of the fundamental organizational similarities in these proteins, they are very different in their properties. Wool is characterized by long range elasticity and moderately low strength. Silk and collagen possess greater strength but a much lower range of elasticity.

The explanation for these vast differences in behavior must reside in the fundamental organizational characteristics such as the nature and structural arrangement of the molecules, because the physical properties of space polymers in general may be considered to depend upon three fundamental structural factors, namely; (a) the character of the main chains; (b) the character of the cross-links; (c) the number of cross-links (19). The physical properties of fibrous proteins may, therefore, be attributed to differences in (a) the amino acid content and their sequence in the main chains; (b) the nature of the side chains; (c) the number and nature of the forces of cohesion between the chains; and (d) the configuration of the chains.

The wool fiber consists of a large number of high molecular weight amino acids of the type which gives rise to large bulky side chains constituting the R groups which vary greatly in size, symmetry, complexity, and functionality, from the hydrogen atom of glycine to the bicyclic structures of tryptophane (3). Close packing of such chains would be unlikely and it would be expected that such fibers would possess low tensile strength. This theory is supported in fact by experiment (14).

Astbury (1) discovered that wool fibers in the normal unstretched condition yield an X-ray fiber diagram the pattern of which is not at all sharp ($\alpha$ keratin). There is a great deal of background diffusion and the photograph is difficult to analyze. Furthermore, wool exhibits a high degree of reversible extensibility intermediate between that of fibers and rubbers, and can be doubled in length without molecular slippage.

The X-ray pattern changes greatly when wool fibers are held stretched to about double their normal length, and has an identity spacing of 3.4 Å (beta keratin). In beta keratin three successive amino acid residues represent a length of 10.2 Å which is just double the 5.1 Å fiber period of the alpha keratin state. This suggests that the alpha keratin is a chain structure in which a three peptide unit has become folded, or spiralled, or both, in such a way that its length is half that of the extended state.

Brown and Harris (4) state that a fiber made up of coiled or folded flexible molecules would be expected to have very low tensile

strength and it can be demonstrated that this would be the case with wool were it not for a unique molecular structure - that is, the presence of covalent disulfide links between the main polypeptide chains. These cross-links have the important function of strengthening the fibers especially in the wet state.

In direct contrast, the structure of silk is uniquely simple as to chemical composition. It is built mainly of the four amino acids, glycine, alanine, serine, and tyrosine and more than 80 per cent of the amino acid residue weight is made up by the three amino acids of low molecular weight (9).

Silk yields an X-ray fiber diagram, an analysis of which shows that the micelle is small and from its dimensions it is indicated that the crystalline portions are built up of glycyl and alanyl units. The fiber period of silk is 7.0 Å. This is substantially what it would be for the fully extended linear glycyl-alanyl chains. The absence of bulky side chains permits close packing and lends itself to extensive hydrogen bridging between the chains. This accounts for the high tensile strength; the extended condition of the chains, and the relatively high degree of crystallinity accounts for the relatively low reversible extensibility compared with that of wool.

A complete review of the structural chemistry of collagen is beyond the scope of this paper. Very excellent reviews are available elsewhere in the literature (15)(2). There are, however, a few pertinent facts, relative to this discussion, which should be included.

The amino acid content of collagen is quite different from that

of both silk and wool and from the standpoint of the bulkiness of the side chains may be considered to be intermediate between them. A few of the residues occur in outstanding proportions, 64 per cent of the total being nonpolar, and consisting primarily of glycine, alanine, and proline. Hydroxylic residues, predominantly hydroxyproline and serine comprise about 16 per cent of the molecular length and the acidic, amide, and basic residues supply the remaining 20 per cent of the side chains.

It is generally agreed that in order to account for the relative nonextensibility of the collagen fibril there must be intense bridging between the chains.

McLaughlin and Theis (15) point out that in collagen, two types of cohesive forces must exist; namely, (a) electrovalent or salt-like linkages and (b) the coordinate or hydrogen bond, the hydrogen bonds being the most prominent.

One of the most characteristic properties of collagen is its shrinkage temperature. When heated, collagen fibers contract suddenly at a temperature which is dependent upon previous treatment and upon the medium in which the heating is carried out. Using water as the heat transfer medium and steer hide collagen this temperature is approximately 60 to 65 degrees C.

Wilson (23) has pointed out that the so-called hydrogen bonds are extremely stable under ordinary conditions but when the protein becomes suddenly swollen by solutions of acids or alkalies, or by the application of hot water, rupture of the hydrogen bonds occur and the

initially stable structure is lost. The shrinkage temperature has long been used as a criterion for tannage since the shrinkage temperature of a tanned sample of collagen is increased by an amount which is characteristic of the particular type and extent of tannage.

Various types of tension studies both in the wet and the dry states have been the most fruitful methods for qualitative and quantitative evaluation of interchain forces in fibers. This dissertation presents some evidence to substantiate the theory that: (a) the treatment of collagen with glyoxal does introduce new and stronger cross-links between the polypeptide chains, (b) the introduction of such cross-links increases the hydrothermal stability of collagen, and (c) the introduction of such cross-links increases the ultimate strength of the fiber as measured by the mean breaking length.

## EXPERIMENTAL

Since the isolation of fibers from the skin after glyoxal tannage was not considered feasible for this work because of the difficulty in obtaining them in sufficient quantity and dimensional uniformity, it was decided to use kangaroo tail tendons. These tendons are readily obtainable because of their use in the manufacture of surgical sutures, and have been used by others for tension studies (10)(5)(11).

The close similarity of collagen from this source to that of skin collagen has been indicated on the basis of the amino acid content by Neuman (16). It is also stated by Highberger (10) that they are composed of nearly pure collagen, and give a very sharp X-ray fiber pattern identical with that given by other collagenous tissues.

The dry fibers were cut to lengths of 8 inches (20.3 cm.) and divided into four groups, hereafter referred to as Groups A, B, C, and D. Each group consisted of 100 fibers weighing 16.5 to 17.0 grams and having diameters ranging from 0.5 to 1.5 mm. The dry fibers were placed in large test tubes of such dimensions that it was possible to obtain a small solution to collagen ratio for the subsequent chemical treatments. The fibers remained in these tubes throughout the processing. The dry fibers were rehydrated by soaking in distilled water for 24 hours, then in 10 per cent sodium chloride solution for another period of 24 hours to remove the globular proteins. At the end of the sodium chloride treatment, they were washed in successive changes of distilled water until free of salt, then air dried. The fibers were stored in the dry

state until such time as the chemical treatments were continued in preparation for the tension testing.

Tanning Procedure

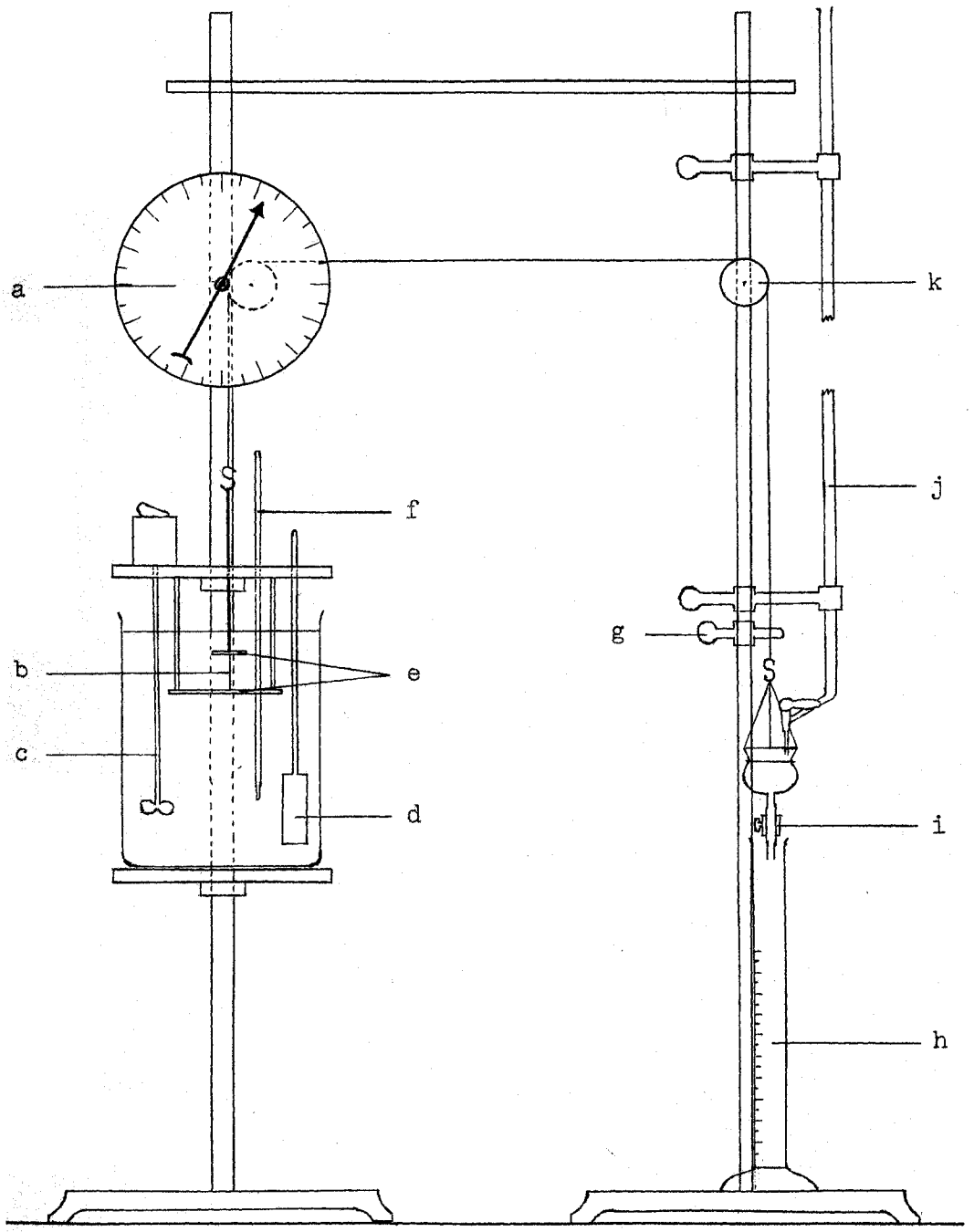
Group A of the series served as the control group, and the fibers were split and tested according to a procedure to be described later. Groups B, C, and D were then treated at room temperature for 24 hours, with occasional agitation, with a 0.1 M $Na_2HPO_4$ buffer containing 0.5, 0.75, and 1.0 per cent glyoxal respectively on the solution basis. The ratio of tanning solution to collagen was 4 to 1. The pH of the tanning solution had previously been adjusted to 7.5 which according to Gustavson (6) is within the range of the maximum stabilizing effect of glyoxal on collagen. At the end of the tanning period, the fibers were washed, air dried, and stored in this state until tested.

The method of tensile testing consisted of a determination of the Mean Breaking Length, a method the applicability of which had previously been confirmed (10)(5)(11). The method eliminated the necessity for determining the cross-sectional area. The results are expressed in terms of the length of the fiber that would break under its own weight if it were suspended from one end.

Apparatus

The apparatus used in the testing consisted essentially of a modified Theis shrinkage meter (15) set up expressly to perform this series of experiments. The apparatus is pictured diagrammatically in Figure I. It was our opinion that more accurate information concerning

FIGURE I




Legend

a. Dial
b. Test sample
c. Stirrer
d. Heater
e. Clamps
f. Thermometer
g. Clamp
h. Graduated cylinder
i. Stopcock
j. Burette
k. Pulley

the molecular forces of cohesion could be ascertained by utilizing a method of wet tension testing than by the dry method (13). The apparatus was, therefore, constructed with this principle in mind.

The apparatus was adaptable for the determination of the elongation at any load as well as the mean breaking length. It consisted of a system for loading and unloading the free end of a fiber at a constant rate while the opposite end was held rigidly in a clamp.

Method of Measurement

The test specimen (b) is held at one end by a clamp rigidly attached to an immovable bar (e). The other end is also held by a clamp but it is free to move in the direction of a force applied during the determination. A flexible cotton covered wire (Dennison's) is attached to the movable clamp and passes over the pulley of a dial system (a) some distance above the test specimen, then across to a frictionless pulley (k), then down to the head of a thistle tube to which it is attached. The thistle tube head is equipped with a capillary tube and clamp (i) to provide for unloading the specimen.

The immovable clamp is attached to a plate which in turn is clamped to a metal stand. The plate is equipped with openings through which pass the shaft of a stirrer (c), a thermometer (f), an immersion heating unit (d), and the shaft to which the movable clamp is attached. This entire mechanism is immersed in water by raising a water filled 2000 ml. beaker over it.

The dial (a) was taken from a Theis shrinkage temperature meter

and consists of a pointer activated by gears attached to the pulley. The gear ratio is so standardized that a movement of the pointer through one graduation on the dial represents a movement of the free clamp through 1/30 mm. By this arrangement, the elongation or retraction of the sample could be recorded for each addition or removal of load. Constant rate of loading was obtained by allowing the mercury to flow from a burette into the free-moving thistle tube head, with the stopcock (i) in the closed position. Constant rate of unloading was obtained by allowing the mercury to flow from the thistle tube head through a capillary tube by opening the stopcock (i). The bore of the capillary was of such diameter as would permit the mercury to drain at a slow, constant rate when the stopcock was fully opened. A hook was attached to the ringstand just above the thistle tube head to catch the mercury loaded container at the instant the fiber broke. Load readings during the unloading process were made by allowing the mercury to flow into a 10 ml. graduated cylinder. The readings in ml. of mercury were converted to grams by a conversion table prepared for this purpose.

The disturbance to the equilibrium of the system caused by opening and closing the stopcock (i) were such that a clamp (g) was installed above the thistle tube head in such a way that when closed it acted as a brake preventing the movement of the wire and thus preventing the length of the sample from changing.

The two sections of the apparatus were rigidly attached to each other and braced in order to prevent the movement of one part of the

system with respect to the other. To the fiber end of the movable system was attached the necessary amount of lead sinkers to properly counter-balance the system.

Even with the use of a wire connection between the load and the fiber there was a sufficient amount of stretch in the wire that a standardization curve had to be run periodically. A heavy steel wire was placed between the clamps, the system was then loaded in the usual way. All stress-strain data was corrected at each load by an amount determined during the standardization procedure.

Considerable difficulty was experienced in holding the fibers in the clamps. Attempts were made to adjust the clamps to overcome this problem. Finally it was found necessary to attach small pieces of oak-tag paper to the clamps in such a manner that the clamp when closed would press the fiber tightly against the paper. This process proved to be very satisfactory and served to hold the fibers rigidly between the clamps.

The word fiber as used in this paper refers to gross fibers ranging in diameter from 0.5 mm. to 1.5 mm. For testing purposes, these gross fibers were split repeatedly until fiber aggregates measuring 0.005 mm. to 0.015 mm. in diameter were obtained.

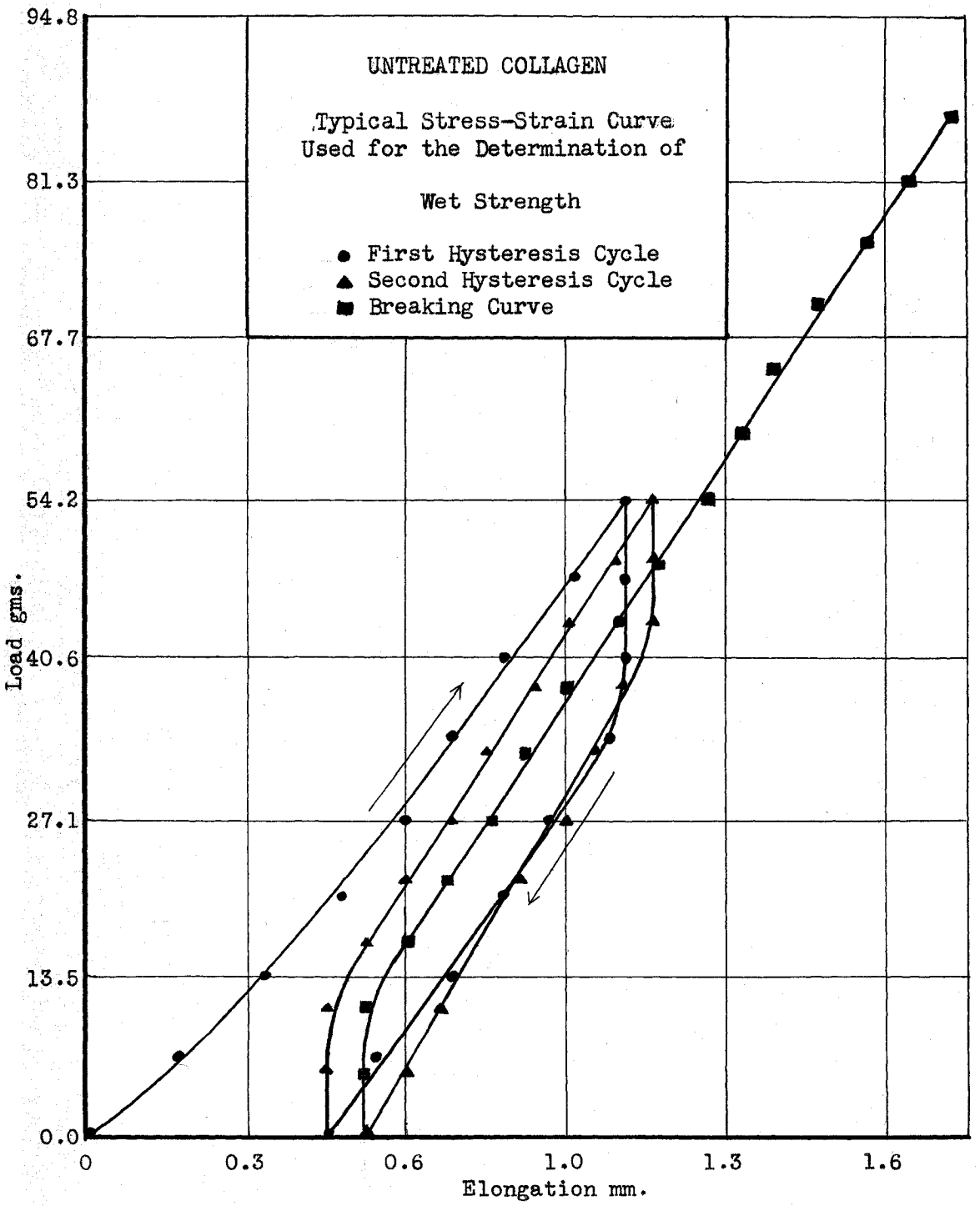
The fiber aggregates were then placed between the clamps of the apparatus. The distance between the clamps was standardized for each determination at 30 mm. as measured with a vernier caliper. The fiber aggregates were then immersed in water at room temperature and allowed to soak for at least 15 minutes. A final adjustment of the length of

the fiber aggregates was made and two hysteresis curves were run, i. e. the fiber aggregates were loaded and unloaded twice at a rate of 0.5 ml. of Hg (6.77 gms.) per minute to a maximum load of 4 ml. of Hg (54.18 gms.). The maximum load of 4 ml. of Hg for the hysteresis loops was arbitrarily set from the data obtained in preliminary tests to be well within the breaking load of the fiber aggregates. Readings of the elongation or retraction were taken at each 0.2 ml. of Hg increment or decrement. The purpose of the first cycle was to straighten out the naturally occurring kinks from the fiber and to obtain a uniform state of tension on the fiber aggregates. A second loading and unloading cycle was then immediately carried out in a manner identical with the first. In the third loading operation, the fiber aggregates were loaded in a manner identical to the loading portions of the previous cycles, however, loading was continued this time until the fiber aggregates broke. A typical curve obtained by the procedure described above is shown in Figure II.

The broken ends of the fiber aggregates were immediately cut from each clamp with a razor blade, blotted with filter paper to remove excess water, placed in a weighing bottle, and weighed on a microbalance. This weight was recorded as "wet weight." The fiber aggregates were air dried for several days, then dried in a vacuum oven at 75 degrees C. over night. They were again finally weighed on a microbalance . This weight was recorded as "dry weight."

From the data so obtained the mean breaking length of the fiber aggregates was calculated in accordance with the following formula (13).

FIGURE II



$$\text{MBL (km.)} = \frac{\text{Length of fiber aggregates (mm.)}}{\text{Weight of fiber aggregates (mg.)}} \times \text{Breaking Load (kg.)}$$

Calculations of mean breaking length indicative of the wet strength were made both on the dry weight and the wet weight basis and the results are recorded in tables I, II, III, and IV.

Shrinkage Temperature Determinations

The shrinkage temperature determinations were made by a micro-method. A small piece of the fiber aggregates about 1 mm. in length was placed in the concavity of a glass slide, the concavity was filled with water and covered with a cover slip. The slide was placed on a Walton microscope warm plate and heated at a rate of 3 degrees C. per minute. The temperature at which the ends of the fibrils began to curl was recorded as the shrinkage temperature. Shrinkage temperatures by this method always result in lower readings because otherwise invisible movement is observed by the aid of the microscope (20).

Since it was indicated by the mean breaking length determinations that the strength of the fiber aggregates was increased by the uptake of glyoxal and since this increase in strength is assumed to be due to the formation of cross-linkages between the polypeptide chains, it was desired to apply the theory of rubber-like elasticity to the gross fibers which remained from the glyoxal treatment. It has been shown by Wiederhorn and Reardon (21)(22) that thermally contracted collagen obeys the kinetic theory of rubbery elasticity. They assumed collagen fibers to be a polymer network in which the polypeptide chains were bound together at intervals along their length, and by the interpretation of

TABLE I

UNTREATED FIBERS

GROUP A

| Wet Weight mg. | Dry Weight mg. | Breaking Load gms. | Mean Breaking Length * Km. | Mean Breaking Length ** Km. |
|---|---|---|---|---|
| .356 | .293 | 102.95 | 8.68 | 10.54 |
| .417 | .315 | 189.64 | 13.64 | 18.06 |
| .290 | .191 | 73.15 | 7.65 | 11.48 |
| .434 | .235 | 92.11 | 6.36 | 11.76 |
| .361 | .253 | 100.24 | 8.33 | 11.89 |
| .396 | .246 | 97.53 | 7.40 | 11.90 |
| .653 | .319 | 159.84 | 7.34 | 15.02 |
| .711 | .404 | 230.28 | 9.72 | 17.10 |
| .584 | .200 | 108.37 | 5.57 | 16.26 |
| .404 | .211 | 138.17 | 10.25 | 20.02 |
| .361 | .207 | 86.69 | 7.20 | 12.56 |

* Based upon wet weight
** Based upon dry weight

Statistical Analysis

| * | |
|---|---|
| Number of samples | 11 |
| Arithmetic mean | 8.37 |
| Variance | 4.91 |
| Standard Deviation | 2.22 |

| ** | |
|---|---|
| Number of samples | 11 |
| Arithmetic mean | 14.23 |
| Variance | 10.21 |
| Standard Deviation | 3.20 |

TABLE I
continued

UNTREATED FIBERS

GROUP A

| Area #1 * sq. in. | Area #2 ** sq. in. | Difference between Areas #1 & 2 sq. in. | % Elongation at break | Shrinkage Temperature Degrees C. |
|---|---|---|---|---|
| 5.19 | 3.06 | 2.13 | 8.10 | 52 |
| 2.81 | 2.48 | 0.33 | 8.40 | 54 |
| 4.09 | 3.65 | 0.44 | 4.66 | 54 |
| 4.83 | 2.99 | 1.84 | 7.93 | 54 |
| 3.95 | 2.38 | 1.57 | 6.76 | 51 |
| 4.19 | 1.75 | 2.44 | 6.06 | 53 |
| 2.81 | 2.15 | 0.66 | 6.23 | 48 |
| 2.97 | 2.17 | 0.80 | 8.66 | 50 |
| 4.26 | 3.40 | 0.86 | 7.93 | 53 |
| 3.73 | 2.46 | 1.27 | 7.06 | 52 |
| 4.68 | 2.19 | 2.49 | 5.23 | 54 |

52.3

* First hysteresis loop
** Second hysteresis loop

TABLE II

0.5% GLYOXAL TREATED FIBERS

GROUP B

| Wet Weight mg. | Dry Weight mg. | Breaking Load gms. | Mean Breaking Length * Km. | Mean Breaking Length ** Km. |
|---|---|---|---|---|
| .228 | .146 | 151.72 | 19.96 | 31.17 |
| .283 | .148 | 78.57 | 8.33 | 15.92 |
| .151 | .062 | 48.77 | 9.69 | 23.59 |
| .439 | .174 | 94.82 | 6.47 | 16.35 |
| .434 | .196 | 116.49 | 8.04 | 17.83 |
| .218 | .126 | 92.11 | 12.68 | 21.93 |
| .271 | .140 | 56.89 | 6.30 | 12.19 |
| .518 | .270 | 203.19 | 11.77 | 22.58 |
| .141 | .098 | 59.60 | 12.68 | 18.25 |
| .306 | .146 | 173.39 | 16.99 | 35.63 |
| .380 | .201 | 86.69 | 7.10 | 12.94 |

* Based upon wet weight
** Based upon dry weight

Statistical Analysis

| * | |
|---|---|
| Number of samples | 11 |
| Arithmetic mean | 10.91 |
| Variance | 19.77 |
| Standard Deviation | 4.45 |

| ** | |
|---|---|
| Number of samples | 11 |
| Arithmetic mean | 20.76 |
| Variance | 53.45 |
| Standard Deviation | 7.31 |

TABLE II
continued

0.5% GLYOXAL TREATED FIBERS

GROUP B

| Area #1 * sq. in. | Area #2 ** sq. in. | Difference between Areas #1 & 2 sq. in. | % Elongation at break | Shrinkage Temperature Degrees C. |
|---|---|---|---|---|
| 4.16 | 2.98 | 1.63 | 11.26 | 60 |
| 3.94 | 2.34 | 1.60 | 5.40 | 59 |
| 8.22 | 7.04 | 1.18 | 8.0 | 59 |
| 3.12 | 2.53 | 0.59 | 5.50 | 63 |
| 4.14 | 2.67 | 1.47 | 7.23 | 62 |
| 4.51 | 3.22 | 1.29 | 7.43 | 63 |
| 5.69 | 3.54 | 2.15 | 5.56 | 62 |
| 3.29 | 1.77 | 1.52 | 10.66 | 63 |
| 7.04 | 4.94 | 2.10 | 7.66 | 60 |
| 3.39 | 2.01 | 1.38 | 8.90 | 62 |
| 3.66 | 2.27 | 1.39 | 5.60 | 63 |

* First hysteresis loop
** Second hysteresis loop

61.5

TABLE III

0.75% GLYOXAL TREATED FIBERS

GROUP C

| Wet Weight mg. | Dry Weight mg. | Breaking Load gms. | Mean Breaking Length * Km. | Mean Breaking Length ** Km. |
|---|---|---|---|---|
| .360 | .204 | 176.098 | 14.67 | 25.89 |
| .213 | .130 | 86.694 | 12.21 | 20.00 |
| .212 | .098 | 102.950 | 14.57 | 31.51 |
| .262 | .149 | 143.588 | 16.44 | 28.91 |
| .233 | .115 | 124.623 | 16.05 | 32.51 |
| .241 | .105 | 149.006 | 18.55 | 42.57 |
| .260 | .127 | 108.368 | 12.51 | 25.59 |
| .298 | .178 | 138.169 | 13.91 | 23.28 |
| .154 | .097 | 113.786 | 22.17 | 35.19 |
| .129 | .101 | 75.858 | 17.64 | 22.53 |
| .298 | .191 | 108.368 | 10.90 | 17.02 |

* Based upon wet weight
** Based upon dry weight

Statistical Analysis

| * | |
|---|---|
| Number of samples | 11 |
| Arithmetic mean | 15.42 |
| Variance | 10.45 |
| Standard Deviation | 3.23 |

| ** | |
|---|---|
| Number of samples | 11 |
| Arithmetic mean | 27.73 |
| Variance | 54.37 |
| Standard Deviation | 7.37 |

TABLE III
continued

0.75% GLYOXAL TREATED FIBERS

GROUP C

| Area #1 * sq. in. | Area #2 ** sq. in. | Difference between Areas #1 & 2 sq. in. | % Elongation at break | Shrinkage Temperature Degrees C. |
|---|---|---|---|---|
| 3.59 | 2.50 | 1.09 | 9.33 | 69 |
| 3.98 | 3.11 | 0.87 | 5.76 | 68 |
| 4.22 | 2.85 | 1.37 | 8.00 | 66 |
| 3.66 | 2.22 | 1.44 | 9.20 | 68 |
| 4.37 | 2.80 | 1.57 | 8.86 | 66 |
| 4.43 | 2.71 | 1.72 | 10.03 | 67 |
| 5.52 | 3.12 | 2.40 | 8.93 | 66 |
| 4.42 | 2.96 | 1.46 | 9.70 | 65 |
| 5.13 | 3.36 | 1.77 | 10.03 | 65 |
| 7.39 | 4.13 | 3.26 | 8.26 | 68 |
| 4.40 | 2.25 | 2.15 | 7.26 | 66 |

* First hysteresis loop
** Second hysteresis loop

66.8

TABLE IV

1.0% GLYOXAL TREATED FIBERS

GROUP D

| Wet Weight mg. | Dry Weight mg. | Breaking Load gms. | Mean Breaking Length * Km. | Mean Breaking Length ** Km. |
|---|---|---|---|---|
| .198 | .168 | 116.495 | 17.65 | 20.80 |
| .135 | .114 | 56.893 | 12.64 | 14.97 |
| .144 | .131 | 81.276 | 16.93 | 18.61 |
| .284 | .235 | 105.658 | 11.16 | 13.49 |
| .270 | .249 | 170.679 | 18.96 | 20.56 |
| .310 | .231 | 135.460 | 13.10 | 17.59 |
| .202 | .142 | 138.169 | 20.52 | 29.19 |
| .432 | .252 | 298.012 | 20.69 | 35.46 |
| .139 | .111 | 86.694 | 18.71 | 23.43 |
| .100 | .072 | 59.602 | 17.88 | 24.83 |
| .361 | .239 | 195.062 | 16.21 | 24.48 |

* Based upon wet weight
** Based upon dry weight

Statistical Analysis

| * | |
|---|---|
| Number of samples | 11 |
| Arithmetic mean | 16.76 |
| Variance | 10.23 |
| Standard Deviation | 3.199 |

| ** | |
|---|---|
| Number of samples | 11 |
| Arithmetic mean | 22.12 |
| Variance | 40.52 |
| Standard Deviation | 6.37 |

TABLE IV
continued

1.0% GLYOXAL TREATED FIBERS

GROUP D

| Area #1 * sq. in. | Area #2 ** sq. in. | Difference between Areas #1 & 2 sq. in. | % Elongation at break | Shrinkage Temperature Degrees C. |
|---|---|---|---|---|
| 4.10 | 3.00 | 1.10 | 7.93 | 68 |
| 6.19 | 3.68 | 2.48 | 5.10 | 66 |
| 6.20 | 3.63 | 2.57 | 7.83 | 67 |
| 4.41 | 2.40 | 2.01 | 6.33 | 66 |
| 3.20 | 2.06 | 1.14 | 8.76 | 67 |
| 2.59 | 2.53 | 0.06 | 5.76 | 69 |
| 3.57 | 2.44 | 1.13 | 11.60 | 66 |
| 3.16 | 1.83 | 1.33 | 8.93 | 66 |
| 4.87 | 3.38 | 1.49 | 9.86 | 66 |
| 7.67 | 4.36 | 3.31 | 7.20 | 67 |
| 3.09 | 2.15 | 0.94 | 9.00 | 67 |

66.8

* First hysteresis loop
** Second hysteresis loop

stress-strain curves in terms of rubber elasticity theory were able to determine the average molecular weight between the points of attachment. They further show that this procedure is a valid method of determining the molecular weight of collagen since the results obtained are in good agreement with the molecular weight determinations by calculation from X-ray data and ultracentrifuge methods.

The mathematical relation correlating the force of retraction with the elongation of the sample is as follows:

$$M_c = \frac{V_2^{1/3}}{f} R\ T\ \rho\ (\alpha - \frac{1}{\alpha^2})$$

where

$M_c$ is the molecular weight between cross chains.

$V_2$ is the volume fraction of the rubber constituent in the sample.

f is the retractive force per unit cross-section.

R is the gas constant ($8.478 \times 10^4$).

T is the temperature (absolute).

$\rho$ is the density of the unswollen sample (1.3)(1).

$\alpha$ is the ratio of the stretched length to the initial length.

A number of the gross fibers from each of the groups A, B, C, and D were placed in boiling water for 2 minutes; then they were plunged immediately into cold water. After cooling, the shrunken fibers were air dried and stored in this state until tested.

Prior to testing, the shrunken fibers were rehydrated by soaking in water for 24 hours and then conditioned at the temperature the test

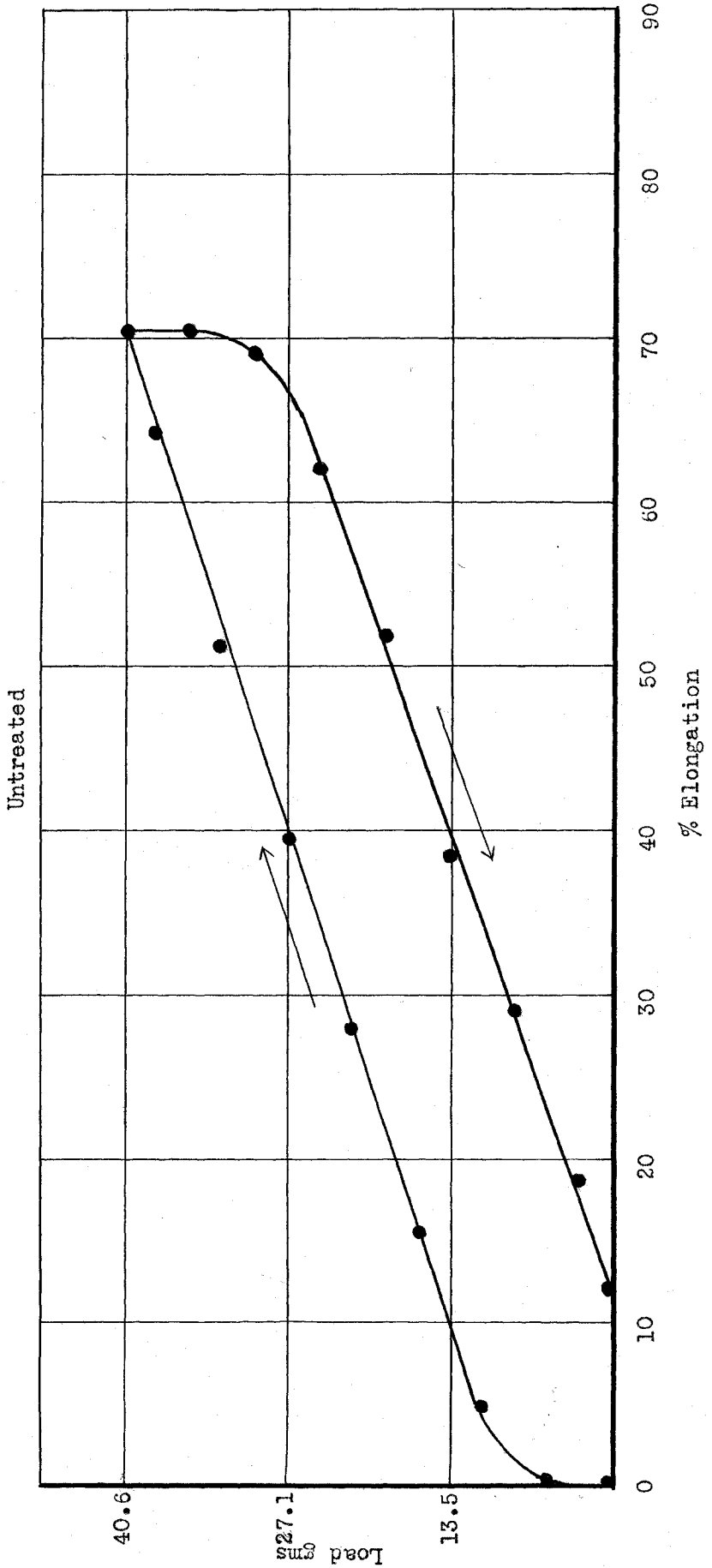
was to be run for 2 hours.

The shrunken gross fibers were then mounted between the clamps of the testing apparatus and adjusted to the standard length of 30 mm. A complete loading and unloading cycle was run with elongation or retraction and load readings taken at each increment or decrement of 0.2 ml. of Hg. Time was permitted after each addition of load for the extension of the fiber to come to equilibrium with the additional load. In order not to stress the fiber to the extent of permanent deformation, the maximum load was 3.0 ml. of Hg (40.64 gms.). The temperature was controlled by a thermoregulator and in each instance was 70 degrees C. ±1 degree C.

At the completion of the stress-strain cycle, the gross fiber was cut from the clamps, quickly blotted with filter paper, and weighed. This constituted the "wet weight." The gross fibers were then dried in acetone for at least 24 hours and finally in a vacuum oven for another 24 hour period and then weighed. This constituted the "dry weight."

From the experimental data thus obtained the average cross sectional area, the volume fraction of the collagen in the swollen sample, and the density of the swollen sample were calculated. The hysteresis curves for each group of gross fibers is illustrated in Figure III. The data and results obtained are recorded in tables V, VI, VII, and VIII. All the calculations were made at the retractive force level of 2 ml. of Hg (27.092 gms.).

FIGURE III A



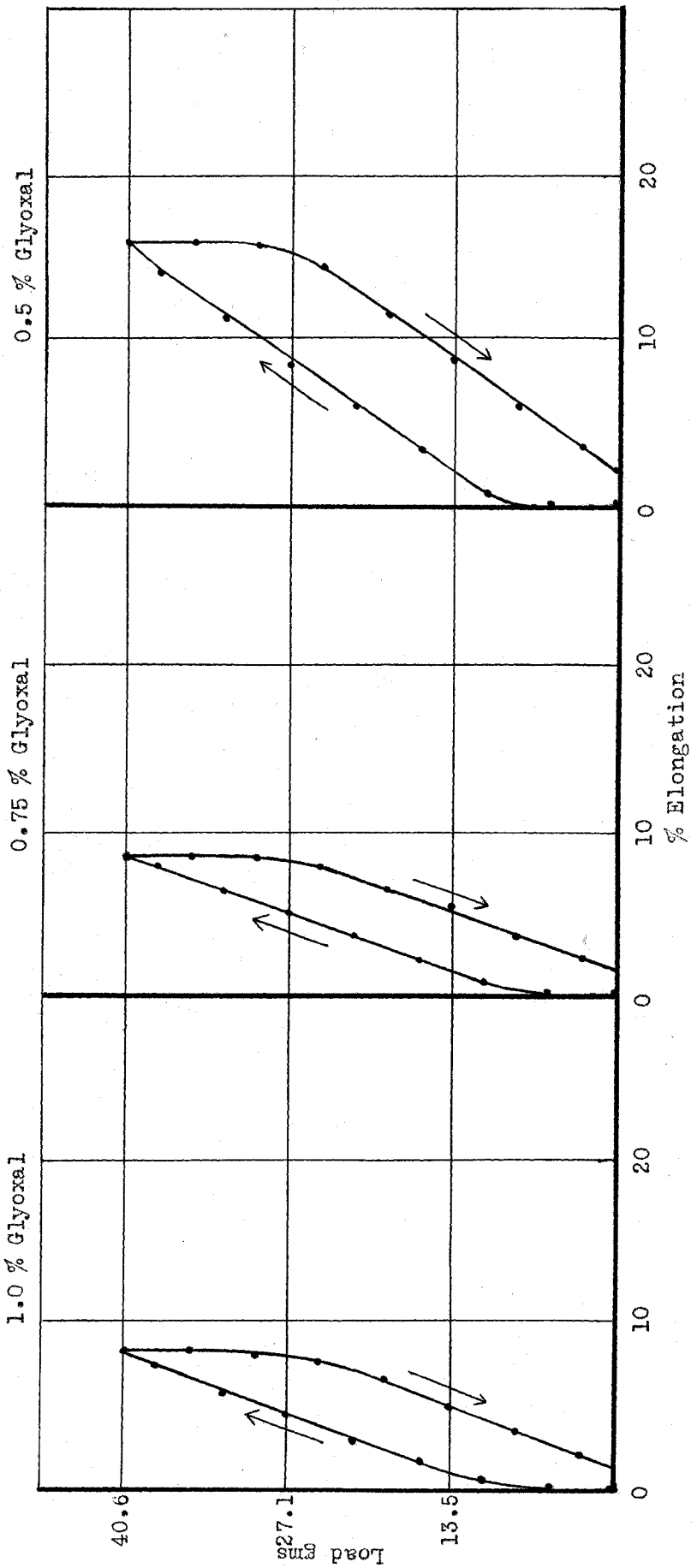



FIGURE III B

TABLE V

MOLECULAR WEIGHT DATA

THERMALLY CONTRACTED COLLAGEN

GROUP A

UNTREATED

| $\alpha - \frac{1}{\alpha^2}$ | $d_2$ | $W_s$ | $V_2$ | $V_2^{1/3}$ | A | F/sq. cm. | $M_c$ |
|---|---|---|---|---|---|---|---|
| 0.831 | 1.03 | 0.005 | 0.15 | 0.53 | 0.119 | 227.85 | 74,200 |
| 0.73 | 1.02 | 0.007 | 0.17 | 0.55 | 0.129 | 210.34 | 78,500 |
| 0.80 | 1.04 | 0.011 | 0.22 | 0.60 | 0.100 | 270.92 | 66,900 |
| 0.99 | 1.01 | 0.005 | 0.13 | 0.51 | 0.138 | 196.32 | 97,300 |
| 1.03 | 1.01 | 0.004 | 0.15 | 0.53 | 0.105 | 258.02 | 79,900 |

TABLE VI

MOLECULAR WEIGHT DATA

THERMALLY CONTRACTED COLLAGEN

GROUP B

0.5% GLYOXAL

| $\alpha - \frac{1}{\alpha^2}$ | $d_2$ | $W_s$ | $V_2$ | $V_2^{1/3}$ | A | F/sq. cm. | $M_c$ |
|---|---|---|---|---|---|---|---|
| 0.151 | 1.06 | 0.014 | 0.32 | 0.68 | 0.077 | 351.84 | 11,200 |
| 0.204 | 1.06 | 0.010 | 0.32 | 0.68 | 0.056 | 483.78 | 10,800 |
| 0.254 | 1.05 | 0.010 | 0.31 | 0.68 | 0.064 | 423.31 | 15,300 |
| 0.306 | 1.05 | 0.007 | 0.30 | 0.67 | 0.045 | 602.04 | 12,600 |
| 0.281 | 1.05 | 0.007 | 0.30 | 0.67 | 0.048 | 564.42 | 12,500 |

TABLE VII

MOLECULAR WEIGHT DATA

THERMALLY CONTRACTED COLLAGEN

GROUP C

0.75% GLYOXAL

| $\alpha - \frac{1}{\alpha^2}$ | $d_2$ | $W_s$ | $V_2$ | $V_2^{1/3}$ | A | F/sq. cm. | $M_c$ |
|---|---|---|---|---|---|---|---|
| 0.162 | 1.07 | 0.007 | 0.38 | 0.72 | 0.033 | 820.97 | 5,200 |
| 0.151 | 1.08 | 0.010 | 0.38 | 0.72 | 0.041 | 660.78 | 5,800 |
| 0.110 | 1.07 | 0.010 | 0.37 | 0.72 | 0.048 | 564.42 | 5,500 |
| 0.119 | 1.07 | 0.011 | 0.39 | 0.73 | 0.053 | 511.17 | 6,100 |
| 0.162 | 1.07 | 0.010 | 0.37 | 0.72 | 0.046 | 588.96 | 7,300 |

TABLE VIII

MOLECULAR WEIGHT DATA

THERMALLY CONTRACTED COLLAGEN

GROUP D

1.0% GLYOXAL

| $\alpha - \frac{1}{\alpha^2}$ | $d_2$ | $W_s$ | $V_2$ | $V_2^{1/3}$ | A | F/sq.cm. | $M_c$ |
|---|---|---|---|---|---|---|---|
| 0.105 | 1.08 | 0.016 | 0.41 | 0.74 | 0.071 | 374.61 | 8,800 |
| 0.110 | 1.07 | 0.010 | 0.42 | 0.75 | 0.045 | 607.44 | 5,200 |
| 0.107 | 1.07 | 0.011 | 0.39 | 0.73 | 0.053 | 515.06 | 6,000 |
| 0.118 | 1.07 | 0.013 | 0.40 | 0.73 | 0.062 | 438.38 | 7,500 |
| 0.196 | 1.07 | 0.010 | 0.39 | 0.73 | 0.047 | 577.65 | 9,400 |

## DISCUSSION AND CONCLUSIONS

The only presently applicable methods for the study of cross-linkages in collagen are those which depend upon such physical characteristics of the protein as tenacity, thermal stability, and stress-strain phenomena. In the present investigation both wet strength and thermal stability measurements were used to show the influence of glyoxal tannage on the cross-linkage of the collagen molecule. The following discussion will indicate the justification for such a choice.

A number of workers have used tensile testing as a method for the quantitative evaluation of interchain forces of various fibrous proteins. In the dry testing of collagen from kangaroo tail tendons, Compton (5) found that tanning decreased the strength of fiber aggregates and, furthermore, in the case of the action of formaldehyde on such collagen he found that increasing formaldehyde concentration in the collagen led to a progressive decrease in strength.

In dry tensile tests Highberger (10) found that tannage decreased the strength of all tannages investigated. He reported also that in the case of formaldehyde tannage, the decrease in strength was the greatest of all the tannages and amounted to about 65 per cent.

In reporting the results of the wet tension testing of collagen gross fibers from kangaroo tail tendons, Jacobson (11) showed a very significant decrease in strength as a result of chrome, vegetable, and formaldehyde tannages. He concludes, "The results of this investigation require, therefore, that the cross-linkage hypothesis of tannage be discarded, or that it be re-examined and re-interpreted."

In an investigation of the influence of tannery processes on the dry strength of collagen fiber aggregates from cattle tendon, Mao and Roddy (13) found that there is no material alteration in fiber strength due to chrome, vegetable, or formaldehyde tannages.

On the other hand, Nutting and his co-workers (17) report that the tensile strength of highly oriented artificial fibers of dry ovalbumen is decreased by formaldehyde treatment but the wet strength is increased by approximately 50 per cent. They report also that the stability of these fibers to water and heat is also improved and that in the instance of the fibers in the fully hydrated state, both mechanical strength and thermal stability are increased by tanning.

Since it is a well known fact that all the commercially used tanning materials affect an increase in the thermal stability of collagen and since this increase in thermal stability is attributable to the introduction of more or stronger cohesive forces between the polypeptide chains, an increase in the thermal stability of collagen should be accompanied by an increase in wet tensile strength. A moderate increase in the density of the cross-linkages should lead to an increase in ultimate tensile strength for the following reasons:

1. The reinforcing action of the cross-links.
2. The reinforcing action attributable to chain alignment and closer packing under tension.
3. The participation of a larger number of chains in restraining the externally applied stress.

Briefly then, if there is an increase in the number of inter-chain

linkages as a result of glyoxal treatment, the wet strength of the treated fibers will be greater than the untreated ones.

It has been found in some systems, that increasing degrees of cross-linkage first results in an increase and later in a decrease in strength characteristics. The loss in strength accompanying the introduction of dense cross-linkages is believed to be due to the following:

1. The interference with chain alignment.

2. The interference with uniform load distribution.

In the present investigation it is shown that the glyoxal treatment resulted in a progressive increase in both thermal stability and tensile strength as a result of an increase in the amount of glyoxal given. The results of this phase of the investigation are shown in tables I, II, III, and IV.

An inspection of the tables will disclose that the strength of the fiber aggregates, as measured by the mean breaking length determinations, increases rapidly and then levels off at the 1.0 per cent level. It will also be apparent that there is a parallel increase in the shrinkage temperature which also levels off at the higher glyoxal level. The shrinkage temperature increase is of the same order of magnitude as that obtained by Gustavson (6) for calf skin.

The apparent discrepancy in the results of the tensile testing studies by the different investigators can readily be explained on the basis of the different techniques used. It should be emphasized that none of the tension studies included the investigation of the effect

of chemical treatment with glyoxal. This bifunctional aldehyde is likely to be quite different in its chemical action from the agents commonly used. Furthermore, the state of hydration and the degree of subdivision of the fibrous units could all be expected to have a profound influence upon the results and the interpretations and conclusions drawn from them. In this connection the following should be considered.

First, the results of dry tension testing studies are not comparable to those of wet tension testing because in the dry fiber the adhesions between the structural units have the effect of greatly increasing the density of the cohesive forces between the chains. This results in an unequal distribution of stress, the entire force being borne by the weakest chains in the unit rather than by the entire network. Furthermore, as stated by Lollar in a discussion of the paper by Roddy (18), "The dry breaking strength undoubtedly reflects the frictional more than it reflects the atomic forces."

Second, the results obtained in the use of gross fibers in tensile testing are not comparable to those of small fiber aggregates. In the use of gross fibers there is likely to be a considerable amount of material present which does not contribute to the strength characteristics, whereas when very small fiber aggregates are present the "diluting" effect is minimized. In a study of tension tests on hide Kanagy, Randall, and Mandel (22) in commenting on the differences between the tensile strengths of chrome and chrome-retanned leather state, "The apparent differences in the results obtained between those two types of leather is that less hide substance (less fibrous material) is con-

tained per unit thickness in the chrome-retanned leather."

Hysteresis curves were run on these fiber aggregates, as mentioned previously, and the areas of the enclosed hysteresis loops, which represents energy dissipated as heat, were measured. As would be expected, in the instance of gross fibers in which only an extremely small amount of stretch and retraction is possible, no definite trend is identifiable. This is undoubtedly largely due to the fact that the varying weights and, therefore, the varying numbers of polypeptide chains participating in the resistance to the stress greatly influences the areas within the curves.

In the second portion of the investigation in which the theory of rubber elasticity is applied to the gross collagen fibers in the thermally shrunken condition, the results of these experiments in tables V, VI, VII, and VIII show a very drastic decrease in the molecular weight of the collagen between cross-chains as the amount of glyoxal given increases. This would appear to be very substantial evidence that new cross-links are being formed as a result of the glyoxal treatment.

Possibly the most convincing evidence in support of the cross-linkage theory in collagen has been supplied by the investigations of Wiederhorn and Reardon (21) and by Wiederhorn, Reardon, and Brown (22) in their work with thermally contracted collagen in both the untreated and the formaldehyde treated condition. They report a molecular weight between the cross chains to be of the order of 55,000 for the untreated collagen, and a range of between 11,000 and 40,000 for the formaldehyde treated collagen, depending upon the formaldehyde uptake of

the fibers.

As outlined in the previous section of this dissertation, the increase in strength and thermal resistance characteristics as a result of glyoxal uptake was attributed to an increasing formation of cross-linkages. Now it is shown that there is a decrease in the molecular weight of collagen between the cross-linkages. The molecular weights ranged from a mean of 79,000 for the untreated fibers to 7,000 in the highest glyoxal concentration used. The decrease is very abrupt even for the lowest concentration and as the amount of glyoxal given increases there is a tendency to level off. This is in agreement with the demonstrated strength characteristics.

In the case of wool keratin, it has been quite clearly shown by the classical experiments of Harris, Mizell, and Fourt (8) that when the -S-S- cross-linkages have been permanently ruptured, the fiber becomes much less resistant to extension. They state, "Much less energy is required to elongate the fiber after the cross-links have been split. ----- The whole scale of the relations of stress to strain has been shifted toward greater extensions for small forces. ----- If, after reduction, the cross-links are largely rebuilt by oxidation of the sulfhydryl groups to the disulfide form, the wool recovers to a large extent its original properties." This work with wool has lent support also to the theory that the vulcanization of rubber involves a cross-linking process.

In view of the fact that Winheim and Doherty (24) in a study of the resin tannage of glyoxal treated collagen claim that glyoxal

binds to collagen by only one aldehyde group, the other participating in resin binding, the question might arise as to how this theory is reconcilable with the views expressed in the present investigation. The fact is that in this study resin tannage was not involved. Furthermore, Highberger in a discussion of the paper by Roddy (18) stated that he is in possession of data which would indicate that about 10 per cent of the formaldehyde in combination with collagen is involved in cross-linkages. There is no reason to suppose that glyoxal would not react in a similar manner.

The hysteresis loops showing the stress-strain properties of thermally contracted collagen, as shown in Figure III, indicate that much less energy is required to elongate the untreated fibers than those having undergone the glyoxal treatment. Furthermore, as the glyoxal uptake increases, the resilient energy is much greater but the hysteresis loop is much smaller. This supplies further evidence to support the thesis that cross-linkages in the untreated thermally contracted collagen, if any, are weak and widely disposed but the formation of cross-linkages can be induced by the chemical treatment of collagen with glyoxal.

From the experimental data presented, it is the author's conclusion that the cross-linking capacity of glyoxal on collagen is a very real chemical phenomenon. The nature of the cross-links and the points of attachment must await further investigation as no information beyond pure speculation is available at the present time.

# BIBLIOGRAPHY


1. Astbury, W. T., J. International Society of Leather Trades' Chemists 24, 78 (1940).

2. Bear, R. S., "Advances in Protein Chemistry", Vol. VII, Academic Press, Inc., New York, (1952).

3. Block, R. J. and Bolling, D., "Amino Acid Composition of Proteins and Foods", Charles C. Thomas, Publisher, Springfield, Ill.

4. Brown, A. E. and Harris, M., Ind. and Eng. Chem., 40, 316 (1948).

5. Compton, E. D., J. American Leather Chemists Association, 44, 140 (1949).

6. Gustavson, K. H., Svensk Kem. Tidskr., 59, 98 (1947).

7. Gustavson, K. H., ibid., 61, 114 (1949).

8. Harris, M., Mizell, L. R., and Fourt, L., Ind. and Eng. Chem., 34, 833 (1942).

9. Harris, M., and Brown, A. E., ibid., 17, No. 6, 323 (1947).

10. Highberger, J. H., J. American Leather Chemists Assn., 42, 493 (1947)

11. Jacobson, K. H., and Lollar, R. M., J. American Leather Chemists Assn., 46, 7 (1951).

12. Kanagy, J. R., Randall, E. B., and Mandel, J. W., "The Fiber Structure of Hides as Indicated by Physical Tests", An Unpublished Report.

13. Mao, Tzu Jen, and Roddy, W. T., J. American Leather Chemists Assn., 45, No. 3 (1950).

14. Meredith, R. J., J. Textile Inst., 36, T 107, T 147 (1945).

15. McLaughlin, G. D., and Theis, E. R., "Chemistry of Leather Manufacture", A. C. S. Monograph Series, Reinhold Pub. Corp., New York, (1945)

16. Neuman, R. C., Arch. Biochem., 24, 289 (1949).

17. Nutting, G. C., Halmer, M., Copley, M. J., and Senti, F. R., Textile Research J., 16, No. 12, 599 (1946).

18. Roddy, W. T., J. American Leather Chemists Assn., 47, 98 (1952).

19. Schmidt, A. X., and Marlies, C. A., "Principles of High-Polymer Theory and Practice," McGraw-Hill Book Co., Inc., New York, (1948).

20. Weiss, H. V., Doctorate Dissertation, University of Cincinnati, (1952).

21. Wiederhorn, N. M., and Reardon, G. V., J. Polymer Science, 9, No. 4, 315 (1952).

22. Wiederhorn, N. M., Reardon, G. V., and Brown, A. R., J. American Leather Chemists Assn., 48, 7 (1953).

23. Wilson, J. A., J. American Leather Chemists Assn., 36, 584 (1941).

24. Winheim, A. H., and Doherty, E. E., J. American Leather Chemists Assn., 45, 122 (1950).