A COMPARATIVE STUDY OF COLLAGEN AND ELASTIN

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by

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TABLE OF CONTENTS

GENERAL INTRODUCTION

The purpose of the present work was to further define what is meant by collagen and elastin by characterizing the properties and determining the amino acid composition of collagens and elastins from a wide variety of sources. The ultimate objectives of determining properties and composition of these proteins are: 1) The utilization of characteristics common to collagens and elastins so that possibly identification and accurate determination can be made of each protein in admixtures of each other and/or with other substances; 2) The determination of any differences in physical, chemical, or biochemical properties in normal or pathological, young or old organisms; 3) The clarification of the biochemistry involved in the formation, maintenance, dissolution and function of connective tissues as well as associated phenomena.

Collagen and elastin are insoluble proteins to which ordinary criteria of purity are not applicable. Histological and questionable chemical tests previously have been used for identification and estimation of these proteins. Often collagens studied have been prepared by procedures which altered their structure and susceptibility to enzyme action.

Properties of collagens and elastins necessarily are intimately associated with structure. Limited observations of differences of properties of so-called collagens from different sources have been made. However, little study has been given these naturally occurring modifications of structure.

Obviously the amino acid composition of the proteins should be investigated as a possible clue to properties and structure. The properties chosen for investigation were:

1) Transition temperatures of collagens from different sources. The transition temperature measured by visual procedures is termed the "shrinkage temperature." Collagens heated in water for several minutes at 45-70° C. undergo a visible contraction and distortion of structure (1, 2). The x-ray diffraction pattern is made irregular and disoriented (3). The susceptibility to tryptic action is increased (4). These changes are believed due to rupture of hydrogen bond and polar bond cross-linkages between the polypeptide chains of native collagen; the chains then collapse upon themselves and assume a relatively disorganized configuration. Thus the transition temperature is a measure of the instability of the collagen structure.

More direct evidence that hydrogen bonds and polar bonds are broken in heat denaturation is provided by studies

with reagents combining with released groups. Tannins can combine with released basic amino acid groups and with accessible peptide linkages. Denaturation of hide collagen resulted in increased fixation of vegetable tannin. When the basic amino acid groups were inactivated with a syntan (a polymeric naphthalene sulfonic acid), the denatured collagen still showed increased tannin fixation, probably due to combination with peptide linkages no longer forming hydrogen bonds. Chromium fixation is increased by previous swelling, lyotropic salt treatment, and heat denaturation.

Gustavson (5) reports that the shrinkage temperature of calf skin was lowered 29-31° C. by treatment in 0.1 N HCl. The total decrease could not be ascribed to rupture of polar bonds in the collagen structure because the lowering of shrinkage temperature is less in the presence of 5% NaCl which inhibits shrinkage. The observed lowering of shrinkage temperature by 0.1 N HCl was concluded to be due to splitting of polar bonds as well as the splitting of hydrogen bonds by swelling. 0.1 N β-naphthalene sulfonic acid lowered the shrinkage temperature 11° C. This acid releases carboxyl groups of collagen without causing swelling. The decrease in shrinkage temperature may be taken as a measure of the effect of complete rupture of internal salt linkage on hydrothermal stability.

Theis (6) determined the shrinkage temperature in solutions of acid and alkali. He found sharp decreases of shrinkage temperature at below pH 3 and above pH 10. These pH's are close to those at which swelling is maximal. The lowering of shrinkage temperature under this condition is attributed to the breaking of hydrogen bonds.

It is thus generally accepted that heat denaturation of collagen is associated with disruption of polar and hydrogen bonds.

2) Conversion of collagen from various sources to water soluble derivatives by heating in water, 55-100° C. In conversion of collagen to gelatin in hot water, heat denaturation occurs followed within a few minutes or hours by the formation of gelatin. Continued boiling of gelatin solutions for a relatively long period results in a gradual degradation to smaller particles as measured in ultracentrifugal and viscosity studies. These changes are explained by Scatchard and Oncley (7) as due to an initial disruption of hydrogen and polar bonds that occur in heat denaturation and a hydrolysis of widely-spaced easily hydrolyzable bonds in the peptide chain. In this way are formed the high molecular weight polypeptide chains that constitute gelatin. Degradation to smaller particles slowly follows through hydrolysis of less easily hydrolyzable peptide bonds.

3) Swelling of collagens from various sources. According to Lloyd (8) this swelling in acid and alkali solution has been explained satisfactorily by a simple theory, at least, as far as volume and weight changes are concerned. Proteins are ampholytes. At pH values removed from the isoelectric points colloidal ions are formed. In gelatins and protein fibers a membrane equivalent is established, and swelling occurs until internal excess osmotic pressure drawing water into the system is balanced by cohesion forces. Salts repress acid swelling due to suppression of internal osmotic pressure. Salts of the lyotropic series promote swelling in neutral solution by weakening the cohesive forces. As previously discussed swelling apparently is associated with rupture of hydrogen bonds, for at maximal swelling the shrinkage temperature undergoes a sharp decrease.

4) Effect of proteases on native and altered (denatured) collagens and elastins. Certain proteases are said to digest collagen and elastin. Denaturation results in greater susceptibility of collagen and elastin to trypsin. It has also been reported that trypsin pretreatment increases the rate of conversion of collagen to water soluble derivatives by the splitting of appropriate linkages.

5) Amino acid composition of collagens and elastins from different sources. An amino acid peculiar to collagen

or elastin may be utilized in conjunction with other characteristics for determination of these proteins. Physical properties of different collagens may be related to chemical differences. A knowledge of composition may be applied for detection of contaminants. For example, tryptophan apparently is not a constituent of pure collagen or elastin. In addition, histidine and methionine apparently are contaminants of elastin.

Studies of properties and composition of collagen and elastin are justified by several important considerations. Collagen is the principal extracellular fibrous protein occurring in connective and supporting tissues of higher animals. Of the total protein in the body 20-30% is collagen. It permeates all portions of the body: bones, cartilage, ligaments, skin, tendons, loose connective tissue, fascia, aponeuroses, blood vessels, cardiac valves, chordae tendinae, cornea, sclera, and nerves (9). Elastin is the second principal fibrous protein of connective tissue. Elastin occurs in skin, bone, blood vessels, loose connective tissues, and various ligaments and cartilages (9). The processes of wound healing and scar formation intimately involve collagen formation (10). A number of diseases are associated with abnormalities of collagen - - diffuse scleroderma, disseminated lupus erythematosis, rheumatic fever, rheumatoid arthritis, periarteritic nodosa, thromboangiitis obliterans, serum sickness (45, 47). Collagen may be of secondary con-

cern in a number of diseases, such as liver cirrhosis, coronary thrombosis, and arteriosclerosis. Industrially, collagen derivatives, such as gelatin, glue, and leather, are important. Finally, collagen and elastin may serve as food.

Collagen is not confined to mammals; it occurs in corresponding tissues in birds, reptiles, amphibians, fish, squid, and perhaps in many uninvestigated organisms and tissues.

PREPARATION OF COLLAGEN

Collagen is a major constituent of skin and tendons. It is from these tissues that collagen usually has been prepared. In addition, bones, cornea, fascia, and fish bladder have been sources of collagen. Generally, procedures involving several or all of the following steps are used in preparation:

1) Mechanical removal of adherent tissue.
2) Treatment with NaCl solution to remove globulins and other extractable protein.
3) Treatment with alkaline solutions such as NaOH, $Ca(OH)_2$, or Na_2HPO_4 solutions to remove mucoids.
4) Treatment with trypsin to destroy elastin and other residual contaminating protein.
5) Treatment with organic solvents to remove lipids and for dehydration.

Knaggs (11) prepared collagen from horn pith, achilles tendon, and ossein of the ox by treatment for sixty days with N/5 NaOH.

Sadikov (12) prepared collagen from cattle hide by separating adherent tissue, washing, finely pulverizing, agitating with 0.35% NaOH at room temperature for 18 hours, washing, treating with acetic acid, washing, and air-drying.

Küntzel (13) prepared cattle hide collagen by simply splitting off grain and flesh layers and air-drying. The product was said to be 98% collagen.

Bowes and Kenten (14) also prepared cattle hide collagen by a simple procedure. The fresh hide was washed in 10% NaCl and in water, dehydrated with acetone, removed grain and flesh layers, cut the corium into small pieces, degreased in light petroleum, thoroughly washed in distilled water and dehydrated in acetone.

Highberger (15) treated cattle corium with $CaCO_3$ and trypsin at 35° C. for 72 hours, washed, dehydrated with alcohol, air-dried, ground in a Wiley mill, treated successively with acetone and alcohol at 50° C., air-dried, agitated 3 hours with half-saturated $Ca(OH)_2$, washed, treated with 0.007-0.008 N acetic acid at 10-12° C., washed again, dehydrated in cold 90% ethyl alcohol, washed in ethyl ether, and finally air-dried the fibers.

Bergman and Stein (72) prepared collagen from cattle achilles tendon by removing adherent tissue, freezing with dry ice and passing through a grain grinder, treating for 2 weeks at 0° C. with 10% saline, and then with M/15 Na_2HPO_4 at 0° C. for three days, and with water at 0° C. until extract gave negative chloride test. Finally the product was treated with ether for two weeks.

Pirie (16) prepared collagen from cattle cornea by the procedure of Bergman and Stein.

Beek (17) prepared collagen from cattle hide, achilles tendon, and bone by the procedure of Highberger. The bone had previously been decalcified by treatment with N/10 HCl for four months.

Other investigators have used as sources of collagen goatskin (18), rat tail tendon (19), kangaroo tail tendon (20), codfish and eel skin (21), and fish bladder (22).

In the present study mild procedures and reagents were used to preclude denaturation. Trypsin was not used because of doubt regarding its effect on native collagen.

Sources of the collagen were cattle achilles tendon, pig achilles tendon, sheep achilles tendon, rhesus monkey achilles tendon, chicken tarso-metatarsal tendon, cattle bone, cattle tail tendon, rat tail tendon, subcutaneous membrane of the Cumberland turtle (terrapin) Pseudemys elegans, and fish (halibut) skin. These tissues initially

appeared to be chiefly collagen fibers*.

Preparations to be used in the study of enzyme action on collagen and in the study of some of the physical properties of collagen were produced by a simple procedure. By means of scissors, scalpel, and running water, adhering membranes, fat, flesh, blood, and mucus were removed. The tissues were then cut into small pieces and shredded in a Waring blendor in an ice and water medium. The fibers were removed, washed in distilled water, and successively extracted for 8–20 hours in 20 times their weight of acetone, alcohol, and ether at 4° C. The samples were then air-dried.

For the study of the amino acid composition and for certain other studies collagen was prepared by a prolonged extraction of impurities. The shredded tissues from the Waring blendor were washed in distilled water and suspended in 50 times their weight of 10% NaCl solution at 4° C. This solution was renewed several times over a period of two

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*We are grateful for materials which were generously supplied to us as follows:
Kangaroo tail tendon by Johnson and Johnson Co., New Brunswick, N. J., and by Dr. T. Salo of M. I. T.
Steer hide collagen by Dr. Peter Buechler and Dr. Robert Lollar of Tanners' Research Council Laboratory, Cincinnati. Two samples were obtained: one prepared according to a published procedure (15) by using $Ca(OH)_2$ and trypsin, and the second by brief treatments at 0° C. with 10% NaCl and then with acetone.
Sodium "Lorol" sulfate, a wetting agent, supplied by E. I. DuPont de Nemours and Co., Wilmington, Delaware.

weeks. Then the fibers were washed and suspended in 50 times their weight of M/15 Na_2HPO_4 solution at 4° C. for three days. The solution was renewed several times over this period. The Na_2HPO_4 was removed by washing and suspension in distilled water for 24 hours at 4° C. The final extraction solutions for typical preparations such as cattle, pig, sheep achilles tendon, and fish skin, yielded negative Molisch and biuret tests. Finally the fibers were successively treated for 8-20 hours in 20 times their weight of acetone,alcohol, and ether at 4° C. The samples were then air-dried. Study of enzyme action, heat denaturation, and conversion to gelatin in hot water of cattle and pig tendon collagen revealed these properties to be practically the same for the given collagen regardless of the mode of preparation.

The cattle bone collagen preparation required decalcification of the bone. Fresh shank bones were slowly bored under ice water with a power drill. The shavings obtained were decalcified in N/10 HCl at 4° C. over a period of four months as suggested by Beek (17). Frequent changes of the solution were made. Subsequently the prolonged extraction procedure was carried out.

The purity of collagen attained may be difficult to appraise. The usual criteria of purity of proteins are not applicable. However, the preparations may be considered

essentially pure for the following reasons:

1) The initial tissues are reported to be largely collagen fibers (9).

2) The cold extraction treatments were calculated to affect contaminants only. After a time their extractive action declined almost to zero.

3) Conversion to gelatin by autoclaving at 15 pounds for 2 hours left only traces of insoluble residue.

4) The samples were inappreciably susceptible to tryptic digestion.

5) Tryptophan apparently is a contaminant of collagen; it was present in less than 0.01% in all samples tested.

6) Samples were in close agreement in amino acid assays.

7) Direct determination of collagen according to the method of Lowry *et al* (23) yielded values of 90-100%. This method is stated by the authors to give a 10% variation on duplicate samples. Various other uncertainties are incurred in this determination. Collagen from different sources or prepared in different ways may react differently in the various steps of the assay.

Possibilities for Future Development

Development of a sensitive, specific, and reliable assay for collagen is being considered in this laboratory. Such a method would be of value as a criterion of purity of collagen preparations. Studies of occurrence, distribution, and modification of collagen would be facilitated by practicable assay procedures.

PREPARATION OF ELASTIN

Elastin previously has been prepared from cattle ligamentum nuchae and cattle aorta. The problems of purification are removal of adhering tissue, blood, lymph, cellular material, mucoids, collagen, and fat. Mechanical processes are effective to a degree. Appropriate solvents may extract various protein and fat contaminants. Collagen can be removed by conversion to gelatin. Elastin is highly resistant to boiling in water, dilute acids, alkalis, and cold concentrated acids and alkalis.

Horbaczewski (24) in 1882 obtained elastin from ligamentum nuchae by successively grinding, washing in water, boiling in dilute acetic acid, dilute alkali, dilute mineral acid, and finally in water. Richards and Gies (25) improved the preparative procedures by washing the ground ligament 24-48 hours in cool running water, treating 48-72 hours in a large excess of half-saturated $Ca(OH)_2$, boiled in water for 75 hours, boiled in 10% acetic acid for 4 hours, and placed in 5% HCl for 18 hours at room temperature. The residue was washed and treated with boiling 10% acetic acid, cold HCl, water, hot alcohol, and ether.

In 1884 Schwarz (26) prepared elastin from cattle aorta by digestion with pepsin and HCl and by subsequent washing and boiling in water for 6 hours. Bergh (27) prepared cattle aorta elastin similarly but suspended the

pepsin-treated tissue in cold dilute HCl for 24 hours before washing.

The preparation by Stein and Miller (28) in 1938 from cattle ligamentum nuchae apparently embodies improvement over earlier procedures. Fresh cattle ligamentum nuchae was freed from adhering tissue, ground in a meat grinder, mixed with dry ice and passed through a machine feed grinder. The finely ground product was successively treated with cold 5% NaCl, cold water, and M/15 phosphate buffer, pH 8.0. The residue was boiled in water 10-14 days, treated with boiling alcohol, and washed with acetone. Grinding and extraction procedures at low temperatures are for the purpose of extracting protein contaminants without rendering them less soluble by denaturation. Boiling in water was to remove collagen. Treatment with the organic solvents was to remove fatty substances.

Braun (29) reported that proteins accompanying elastin were soluble in boiling 40% urea whereas elastin was not. Stein and Miller (28) boiled a portion of the ground ligamentum nuchae in 40% urea for 4-6 days and obtained a product almost identical in elemental composition and properties to the water-boiled preparation.

The preparations by Stein and Miller were considered quite pure because of the agreement in elemental

composition of the differently prepared samples, application of preparative extraction procedures until effects were negligible, and the resistance to solubilization by various reagents that conceivably would dissolve contaminating materials.

In the present study the following method was used. By means of scissors, scalpel, and running water, adhering membranes, fat, flesh, blood, and mucus were removed. The tissues were cut into small pieces and shredded in a Waring blendor in an ice and water medium. The shredded tissues were washed in distilled water and suspended in 50 times their weight of 10% NaCl solution at 4° C. This solution was renewed several times over a period of two weeks. Then the fibers were washed and suspended in 50 times their weight of M/15 Na_2HPO_4 solution at 4° C. for three days. The solution was renewed several times over this period. The Na_2HPO_4 was removed by washing and suspension in distilled water for 24 hours at 4° C. The tissues then were suspended in 50 times their weight of distilled water and heated in a boiling water bath for 24 hours. The liquid level was maintained constant; frequent changes of solution were made. Also, after the first few hours of boiling, the particles were shredded further in the Waring blendor. A slurry of fine insoluble particles was thus

obtained. In the final stages of this boiling the supernatant liquid was perfectly clear and yielded negative biuret and Molisch tests. The elastin was finally treated twice with 50 times its weight of boiling acetone, and similarly with boiling alcohol, and warm ether. The samples were then air-dried.

In a second purification procedure cattle ligamentum nuchae and cattle, pig, and sheep aorta were shredded and treated with cold solutions as in the previous procedure. The tissues were then suspended in 50 times their weight of 40% urea and heated in a boiling bath for 40 hours. The liquid level was maintained constant. Frequent changes of liquid were made. Early in the heating the particles were reduced to a slurry with the Waring blendor. The final changes of liquid were perfectly clear and yielded negative phosphotungstic and Molisch tests. The elastin was washed thoroughly and treated twice and with 50 times its weight of boiling acetone, boiling alcohol, and warm ether. Finally the samples were air-dried.

For the study of enzyme action on elastin a sample of cattle ligamentum nuchae was shredded and treated with cold solutions as were the other preparations; however, the treatment with hot solutions was omitted.

In regard to the purity of the elastin samples treated at 100° with water or urea it may be said:

1) The samples consisted of light yellow fibrous particles, apparently homogeneous under the microscope.

2) Tryptophan, methionine, histidine, which may be considered contaminants in elastin, were present in traces only. Lysine was present in amounts of a uniformily low order for the different preparations.

3) The purification procedures were calculated to remove contaminating materials without affecting the elastin.

4) Two different procedures yielded samples of similar composition.

Suggestions for Future Work

It is possible that modification of boiling treatments would yield purer products. Appropriate enzymes might be of value in the purification of elastin.

Improved preparation of purified elastin of unaltered properties and composition perhaps would contribute to further characterization of this protein.

THE ASH CONTENT OF COLLAGEN AND ELASTIN PREPARATIONS

To obtain ash content of the preparations 500 mg. of sample were weighed into tared platinum crucibles. The samples were ignited and the platinum crucibles were heated to a dull red until the carbonaceous material was entirely consumed. The crucibles were cooled in a desiccator and weighed. As shown in Table I, ash of all preparations analyzed except cattle hide ranged from 0.04% to 0.26%. The cattle hide contained 0.64% ash.

TABLE I

ASH CONTENT OF COLLAGEN AND ELASTIN PREPARATIONS

Preparation	Ash
	%
Hide Powder*	0.18
Cattle Hide Collagen (Prepd. with $Ca(OH)_2$ and trypsin)	0.64
Cattle Hide Collagen (Simple treatment)	0.46
Cattle Achilles Tendon Collagen (Prolonged treatment)	0.08
Pig Achilles Tendon Collagen (Simple treatment)	0.16
Pig Achilles Tendon Collagen (Prolonged treatment)	0.04
Cattle Bone Collagen (Prolonged treatment)	0.18
Rat Tail Tendon Collagen (Simple treatment)	0.26
Cattle Elastin (Ligamentum Nuchae) (24 hours at 100° C. in water)	0.23

* American Standard Hide Powder, Keystone Tanning and Glue Company, Ridgway, Pa.

THE "COLLAGEN" CONTENT OF COLLAGEN PREPARATIONS

Collagen was determined in a number of the samples by the Lowry method (23). The range of values was 86-100% as shown in Table II. This method is reported by Lowry to give variations of 10% in duplicate determinations. The procedure involves extraction with N/10 NaOH. It has not been determined that such extraction only removes contaminating proteins and not appreciable amounts of collagen. Lower values would result from solubilization of collagen by the N/10 NaOH; for example, fish skin collagen was almost completely dissolved by the treatment with N/10 NaOH.

No method of determining collagen is satisfactory. Now planned by this laboratory is the development of an improved assay procedure for collagen.

TABLE II

"COLLAGEN" CONTENT OF PREPARATIONS
(by method of Lowry)

Preparation	"Collagen" %
Hide Powder	73.6
Cattle Hide Collagen ($Ca(OH)_2$ and trypsin)	96.4
Cattle Hide Collagen (Simple NaCl extraction)	91.7
Cattle Achilles Tendon Collagen (Simple treatment)	95.4
Cattle Achilles Tendon Collagen (Prolonged treatment)	97.8
Pig Achilles Tendon Collagen (Simple treatment)	86.4
Pig Achilles Tendon Collagen (Prolonged treatment)	100.0
Rat Tail Tendon Collagen (Prolonged treatment)	88.2
Fish Skin Collagen (Simple treatment)	1.8

TRANSITION TEMPERATURES OF COLLAGEN

Native collagen heated at moderate temperatures (45-70° C.) in water undergoes visible contraction and distortion of structure (1, 2). The temperature at which this occurs is called the transition temperature or "shrinkage temperature". The effect can be considered heat denaturation of collagen. The modified collagen obtained has lost its regularity of x-ray diffraction (3) and resistance to tryptic action (4). The transition temperature is reported to be 52° C. for fish skin collagen and 60-70° C. for cattle tendon collagen (2) or goat skin collagen (30). It is evident that the transition temperature for collagens from different sources may differ considerably.

It is believed that hydrogen bonds and polar cross-linkages connecting the polypeptide chains of collagen are disrupted at the transition temperatures. The chains fold upon themselves and the organized lattice work is destroyed. The transition temperature is therefore a measure of the stability of the native collagen structure (31).

In the present study a survey is made of the transition temperatures of collagens from several different sources. When the collagens were subjected to the heat treatments in water, shrinkage was quite apparent. However, for quantitative data the increased susceptibility to trypsin was determined. As reported by Wöhlisch (32) rat

tail tendon was almost completely converted to a water-soluble form at 60° C., the shrinkage temperature. This was observed to be true in the present study; hence, this transformation was determined as a measure of the transition temperature.

EXPERIMENTAL

To measure the effects of heating collagen in water, solubilization by heating and by subsequent digestion with trypsin was determined. The collagen samples were prepared by the procedure involving prolonged extraction. A suspension of 50 mg. of air-dried collagen sample and 4.0 ml. of distilled water were placed in 18 x 150 mm. pyrex tubes. After standing 3 hours at room temperature to insure wetting, the tubes were placed in a water bath at designated temperatures (56-66° ± 0.5° C.) for 20 minutes. The supernatant liquid was poured off and 4.0 ml. of 0.05 M phosphate buffer, pH 7.2 and 1.0 ml. of phosphate buffer containing 1.0 mg. of trypsin (Merck 1:250)were added. After incubation at 37° C. for 24 hours, the residues were collected in Gooch crucibles, washed with water and alcohol, dried to constant weight at 100-110° C., and weighed. The data obtained are included in Table III. In Table IV are results from preliminary experiments conducted similarly to those in Table III, but the temperature was less closely controlled.

For Table IV collagens prepared by the simple procedure were used.

RESULTS

The collagen samples displayed a wide range of reaction to the heat treatment. It will be noted from Table III and IV that cattle achilles tendon, sheep achilles tendon, and kangaroo tail tendon collagens were not solubilized by the heat treatment. Pig achilles tendon, cattle tail tendon, turtle collagen, and chicken tarso-metatarsal tendon collagens solubilized to an extent of 20-30% by the heat treatment. Rat tail tendon collagen was almost completely solubilized by heating at 60° C. Even though kangaroo tail tendon was resistant to solubilization, its resistance to tryptic action was reduced to almost zero by heating at 60° C.; other collagens were not denatured so completely at 60° C. Graph I shows the considerable effect of small changes of temperature.

Collagens prepared by the simple procedure and by the prolonged procedures were closely alike in their responses to heat and enzyme treatment.

It is apparent that collagens from different sources differ in their resistance to solubilization and to heat denaturation as measured by susceptibility to trypsin. In the section on the action of proteases on collagen data

are given indicating that susceptibility of cattle achilles tendon is increased to the same extent for trypsin, papain, and Cl. perfringens enzyme preparations.

An air-dried sample of cattle achilles tendon heated in an oven at 100-110° C. for an hour did not show increased susceptibility to trypsin.

Suggestions for Future Work

Future work of value might include:

1) Extension and refinement of observations on carefully purified native collagen from a wide variety of sources.

2) The effects of pH changes and presence of salts and denaturing agents on the various collagens.

3) Correlation, if possible, of the data with structure, chemical, and biochemical properties.

TABLE III

EFFECT OF HEAT TREATMENT OF COLLAGEN ON SOLUBILIZATION AND ON TRYPTIC DIGESTION

Solubilization	Temperature (° C.) 56	57	58	59	60	61	62	63	64	65	66
	% Solubilization										
Cattle Achilles Tendon Collagen											
By heat						0	0	0	0	0	0
By trypsin						25	43	54	64	72	75
Total						25	43	54	64	72	75
Pig Achilles Tendon Collagen											
By heat					8	10	12				
By trypsin					15	49	58				
Total					23	59	70				
Rat Tail Tendon Collagen											
By heat	25	37	56			81					
By trypsin	--	--	--			--					
Total	--	--	--			--					
Kangaroo Tail Tendon Collagen											
By heat			0	0	0	0					
By trypsin			48	89	98	99					
Total			48	89	98	99					

Heat solubilization represents collagen converted to a water-soluble form by the heating in water for 20 minutes.

Tryptic solubilization represents the additional quantity of collagen converted to a water-soluble form by incubation with trypsin (1.0 mg. Merck 1:250) for 24 hours after heat treatment of collagen.

TABLE IV

EFFECT OF HEAT TREATMENT OF COLLAGEN ON SOLUBILIZATION AND ON TRYPTIC DIGESTION

Solubilization	Temperature (° C.) 55	60	62	63	64	65
			% Solubilization			
Cattle Achilles Tendon Collagen						
By heat			0	0		
By trypsin			16	60		
Total			16	60		
Pig Achilles Tendon Collagen						
By heat			10	24		
By trypsin			37	63		
Total			47	87		
Sheep Achilles Tendon Collagen						
By heat		0				
By trypsin		11				
Total		11				
Cattle Hide Collagen ($Ca(OH)_2$ and trypsin)						
By heat		28				
By trypsin		62				
Total		90				
Cattle Tail Tendon Collagen						
By heat			19	26		
By trypsin			49	68		
Total			68	94		
Kangaroo Tail Tendon Collagen						
By heat		0				
By trypsin		95				
Total		95				

TABLE IV
(Contd.)

EFFECT OF HEAT TREATMENT OF COLLAGEN ON SOLUBILIZATION AND ON TRYPTIC DIGESTION

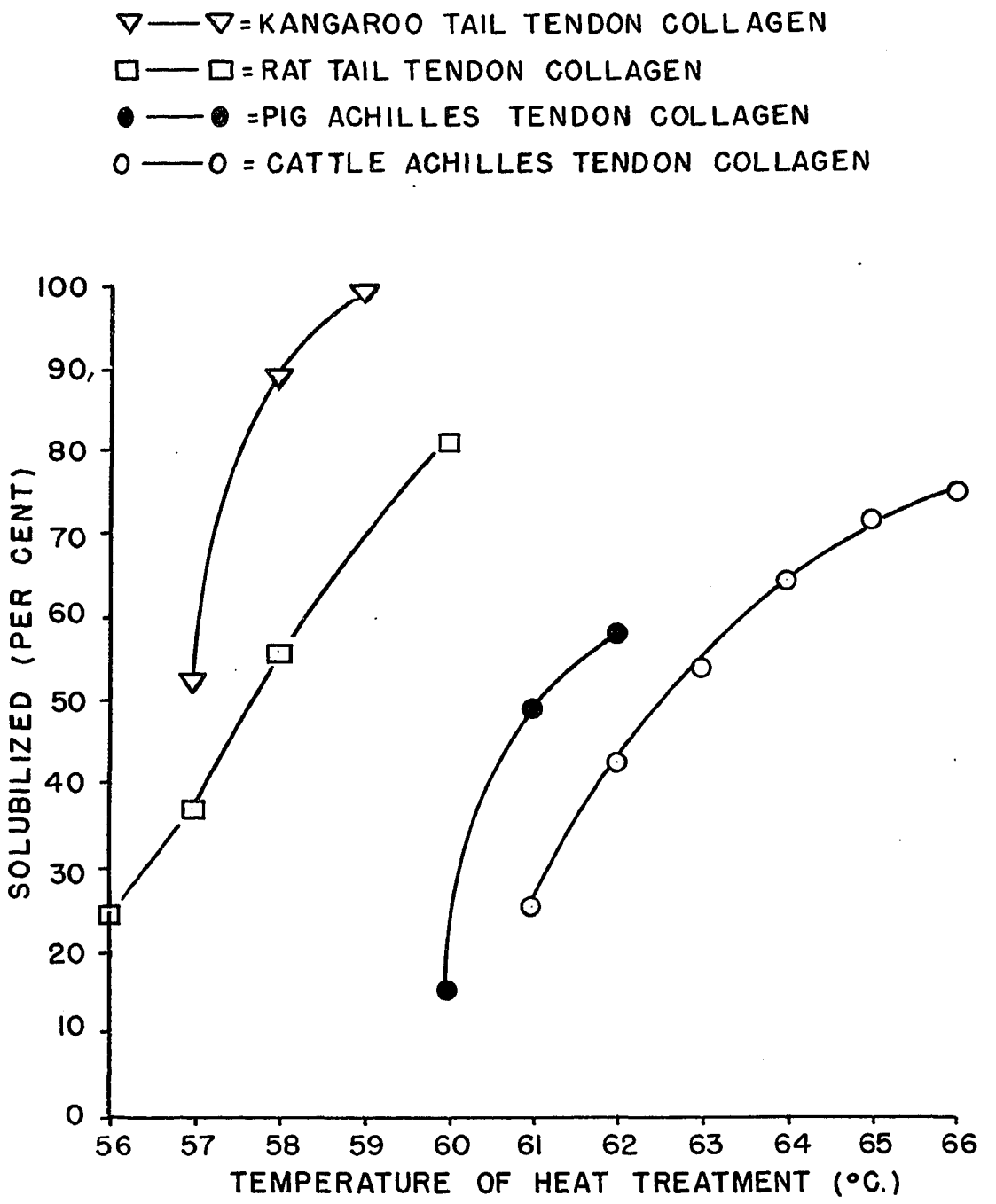
Solubilization	Temperature (° C.) 55	60	62	63	64	65
	% Solubilization					
Turtle Subcutaneous Membrane Collagen						
By heat	1				28	
By trypsin	4				62	
Total	5				90	
Chicken Tarso-metatarsal Tendon Collagen						
By heat						21.5

Heat solubilization represents collagen converted to a water-soluble form by the heating in water for 20 minutes at the specified temperature.

Tryptic solubilization represents the additional quantity of collagen converted to a water-soluble form by incubation with trypsin (1.0 mg. Merck 1:250) for 24 hours after heat treatment of collagen.

Graph I. Transition Temperature of Collagens.

Values are per cent digested by trypsin after heat treatment, except for rat tail tendon collagen for which the values are the per cent converted to a water-soluble form by the heat treatment.



THE SOLUBILIZATION OF COLLAGEN IN WATER

A characteristic property of collagen is solubilization or conversion to gelatin by heating at 55-100° C. in water. In conversion of collagen to gelatin in hot water heat denaturation occurs followed within a few minutes or hours by the formation of gelatin. Continued boiling of gelatin solutions for a relatively long period results in a gradual degradation to smaller particles as measured in ultracentrifugal and viscosity studies. These changes are explained by Scatchard and Oncley (7) as due to an initial disruption of hydrogen and polar bonds that occur in heat denaturation and a hydrolysis of widely-spaced easily hydrolyzable bonds in the peptide chain. In this way are formed the high molecular weight polypeptide chains that constitute gelatin. Degradation to smaller particles slowly follows through hydrolysis of less easily hydrolyzable peptide bonds. The relative ease of solubilization has been used in the present study to indicate differences in collagens from different sources.

EXPERIMENTAL

Collagen prepared by the simple procedure was used except in the case of cattle hide (treated with $Ca(OH)_2$ solution and trypsin), kangaroo tail tendon (air-dried), and hide powder.

Suspensions of 100 mg. of collagen preparations in 5.0 ml. H_2O were contained in 18 x 150 mm. pyrex test tubes. After standing 3 hours to insure thorough wetting, the tubes were placed in a water bath at designated temperature $\pm 1^{\circ}$ C. for 30 minutes. The residues were caught in Gooch crucibles, washed with water and alcohol, dried to constant weight, and weighed. The per cent solubilized was calculated from residue weight.

The solubilization of cattle and pig achilles tendon was further studied in the following manner: Pyrex test tubes (18 x 150 mm.) containing 440 mg. of sample and 10.0 ml. distilled water were sealed by fusion. After standing 3 hours at 25° C. the tubes were immersed in boiling water. At intervals throughout a period of 11.0 hours, tubes were withdrawn and cooled in running water. The liquid was centrifuged or filtered off, and viscosity at 37° C. was determined in an Ostwald viscosimeter. The residues were collected in tared Gooch crucibles, washed, dried to constant weight, and weighed. Nitrogen of the solution was determined by the Kjeldahl procedure. Dialyzable nitrogen of the filtrate was determined by dialyzing 4.0 ml. in cellophane tubing (Visking Corp.) against 16.0 ml. of distilled water in a test tube sealed with paraffined corks. Agitation was produced by 30 end-over-end revolutions per minute in a rotating dialyzer. Phenyl mercuric acetate in 1:100,000

concentration was used as preservative.

RESULTS

It is readily seen from Table V that considerable differences in ease of solubilization exist from one collagen preparation to another. The rat tail tendon collagen was almost completely solubilized at 60° C. (the shrinkage temperature). The kangaroo tail tendon collagen was highly resistant to solubilization even at 100° C. as was cattle achilles tendon collagen. The other samples were intermediate in this property.

In Table VI is a further comparison of the gelatinization of cattle and pig achilles tendon collagen. The amounts solubilized as determined by residue weight and the nitrogen of the supernatant confirmed the contrast in the two collagens. The viscosity of the solutions increased as the nitrogen content increased. The results are also presented in Graphs II and III.

The solubilized cattle collagen was undergoing secondary splitting as indicated by the fall in viscosity in the later stages of heating. 98% or more nitrogen was in particles too large to dialyze through the cellophane tubing. The absolute quantity of dialyzable nitrogen increased gradually as the solubilization progressed.

The residues were minimal after 11 hours of heating the cattle collagen and 3 hours of heating the pig collagen. The collagens from these two sources, prepared by the prolonged extraction procedure yielded relative results similar to those in Table VI.

It is concluded that collagens from different sources prepared in the same manner may exhibit considerably different resistances to conversion to water-soluble forms. These differences may provide information regarding the structure of the collagen molecule.

TABLE V

SOLUBILIZATION OF COLLAGEN AS A FUNCTION OF TEMPERATURE

Collagen Source	Temperature (° C.) 50	60	80	100
	% Solubilized			
Hide Powder	18.9	20.2	25.8	37.6
Cattle Hide (Prepd. with $Ca(OH)_2$ and trypsin)	1.6	2.1	7.3	15.7
Cattle Achilles Tendon		0	1.4	5.3
Pig Achilles Tendon	0	16.1	33.1	36.4
Kangaroo Tail Tendon		0.9	3.5	4.9
Rat Tail Tendon	1.8	81.0	85.4	86.7
Chicken Tarso-metatarsal Tendon		21.5*		

*Heat treatment was 65° C. for 20 minutes.

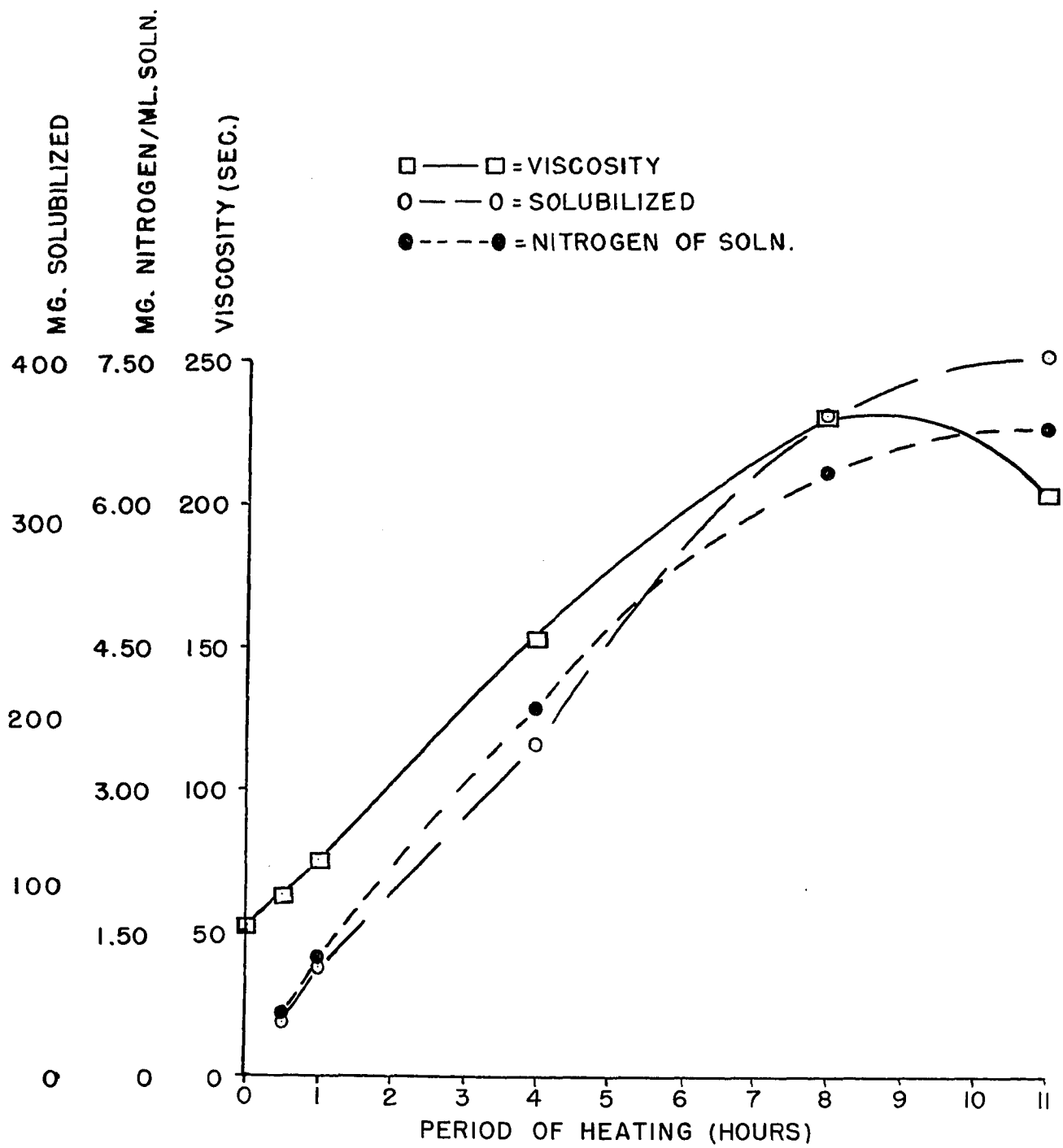
TABLE VI

SOLUBILIZATION OF COLLAGEN AS A FUNCTION OF TIME

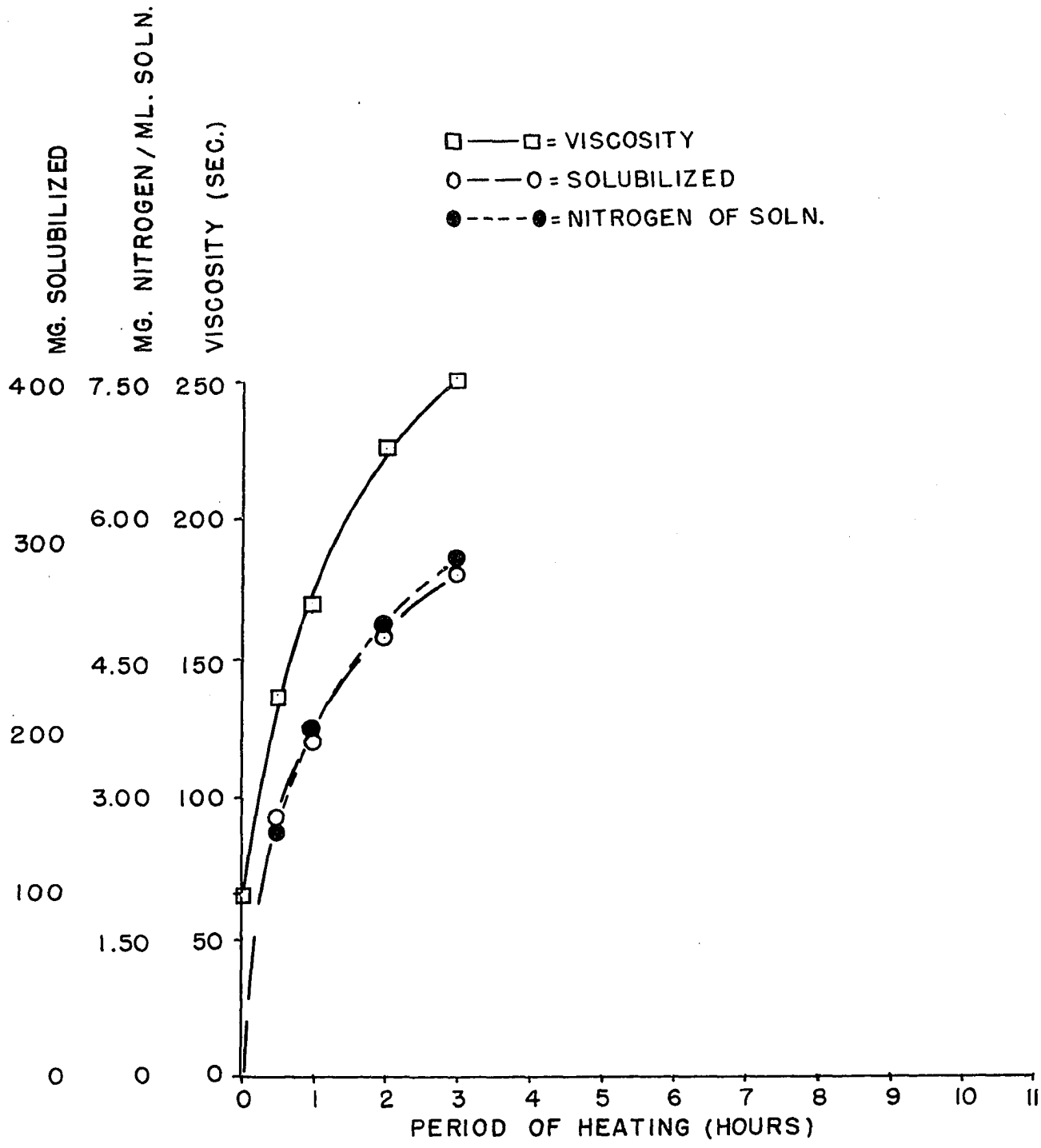
Collagen Source	Time	Solubilized	Nitrogen of Supernatant	Viscosity*	Dialyzable Nitrogen
	(hrs.)	(mg.)	(mg./ml.)	(sec.)	(mg./ml. supernatant)
Cattle Achilles Tendon	0.5	27	0.52	62	0.010
	1.0	59	1.24	74	0.012
	4.0	186	3.96	152	0.013
	8.0	367	6.32	231	0.017
	11.0	378	6.73	202	0.020
Pig Achilles Tendon	0.5	138	2.64	134	0.019
	1.0	193	3.77	171	
	2.0	252	4.84	225	0.013 (?)
	3.0	289	5.58	249	0.072

* Time of flow of distilled water was 56 seconds.

Graph II. The Solubilization of Cattle Achilles Tendon Collagen by Heating at 100° C. in Water.



Graph III. The Solubilization of Pig Achilles Tendon Collagen by Heating at 100° C. in Water.



SWELLING OF COLLAGEN PREPARATIONS

Collagen exhibits marked swelling in dilute aqueous solutions at pH 2-3 and 11-13. In general, a range of minimal swelling extended from pH 5 to pH 10. Gelatin swells similarly as reported by early workers (33,34).

E. C. Porter (35) studied the swelling of hide powder by measuring volume occupied by the settled powder when equilibrium had been established in buffered solutions of different pH values. Two maxima at pH 2.4 and 12.5 were found.

McLaughlin (36) and McLaughlin and Porter (37) did some of the earliest work on swelling in which hide was used. Similar studies have been made by Wilson and Gallun (38), R. E. Porter (39), and Page and Gilman (40). Thickness of strips of hide was a convenient measure of swelling.

Highberger (41) determined swelling of hide collagen by equilibrating with HCl and NaOH solutions and measuring volume after centrifuging as well as measuring weight.

D. J. Lloyd and Mariott (42) determined swelling of rat tail tendon by measuring changes of length and width. A. Küntzel (43) also reported observations on rat tail tendon swelling.

Kernot and Knaggs (44) measured swelling of fish skin.

Swelling of cattle achilles tendon was studied by Knaggs and Schryver (11).

Goat skin swelling was studied by Theis (46).

Swelling of cattle cornea collagen was reported by Pirie (16).

In all these studies the swelling maxima occurred at about pH 2.5 and pH 11-13. A minimum swelling range extended from about pH 5 to pH 10. Swelling of adult rat tail tendon and cattle cornea collagen has a maximum at pH 4. However, it is to be remembered that the swelling of rat tail tendon was determined on pieces of the whole tendon by measurement of length.

In the present study a brief survey was made of the swelling of several collagen preparations as a function of pH.

EXPERIMENTAL

To determine swelling of the collagens as a function of pH, the samples were equilibrated with water, HCl, or NaOH solutions, and the weight of the swollen samples was determined.

The collagen was prepared by the brief treatment described in the section on preparation of collagen. Cattle achilles tendon collagen and pig achilles tendon collagen prepared by the prolonged extraction procedure did not differ significantly from the cruder preparations in extent of

swelling at the region of maximal swelling at pH 2-3.

Suspensions of 100 mg. of air-dried sample in 20 ml. of H_2O, HCl, or NaOH solution representing a range of pH 1 to pH 13 were prepared in 18 x 150 mm. pyrex test tubes. The tubes were stoppered with wax paper and corks. The suspension was maintained with occasional agitation for 24 hours at about 25° C. Swelling is well-advanced within a few minutes and preliminary tests showed ≧4 hours to be sufficient to allow maximal swelling. The equilibrium solution was collected and the pH determined with a Leeds and Northrup pH indicator, No. 7663-A1. The residues were caught in a Büchner funnel over suction and excess fluid pressed out with filter paper. The final residue was only slightly damp to the touch; it was weighed immediately in a tared beaker.

The comparative swelling of heat denatured cattle achilles tendon collagen was determined. The sample in 100 mg. quantities was soaked in 3.0 ml. H_2O for an hour and then placed in a boiling water bath for 5 minutes. Shrinkage occurred in the first minute. According to previous data hydrolysis by this treatment is negligible. The liquid was poured off and the solutions of various pH were added and further procedure followed the usual practice. The dry weight of the various collagen preparations was 85-90% of the air-dried weight.

RESULTS

The data are presented in Table VII and in Graphs IV and V. It may be observed that all samples agreed with each other and with the literature in the position of their maxima and minima. The maxima occurred at pH 2-3 and 12-13. The minima occurred at pH 5 to 10. However, wide variation in extent of swelling occurred. The greatest swelling at the acid maximum took place in the samples more easily solubilized by heating in water.

Pig and chicken collagen swelled considerably more than cattle collagen. Heating at 65° C. for 20 minutes in water solubilized 20% of pig and chicken collagen whereas cattle collagen was not measurably solubilized. Rat and fish collagen which completely hydrolyzed in a few minutes at 60° C. apparently swelled to the point of dissolution in the low pH region. This dissolution may be solubilization similar to that which collagens undergo when heated in water. It is interesting to note that the heat denatured cattle achilles tendon collagen swelled at the acid maximum about 40% as much as the native collagen.

The range of minimal swelling for most of the collagens existed from pH 5 to pH 10. A sudden increase of swelling of rat collagen was observed at pH 4.9. This confirms a previous report by A. Küntzel (43).

It appears that, whatever factors are involved in swelling, there are distinct differences in these factors in collagen preparations from different animals.

TABLE VII

SWELLING OF COLLAGENS AS A FUNCTION OF pH

Initial Solution	HCl N/100	HCl N/1000	HCl N/10,000	HCl N/100,000	Distd. Water	NaOH N/10,000	NaOH N/1000	NaOH N/100
Cattle Achilles Tendon Collagen								
pH of solution at equilibrium	1.25	2.24	2.68	3.93	6.13	10.51	11.77	12.55
Weight of residue (mg.)	879	2600	2676	479	262	329	1322	1246
Cattle Achilles Tendon Collagen (Prolonged treatment)								
pH of solution at equilibrium		2.29			5.54			
Weight of residue (mg.)		2517			215			
Cattle Achilles Tendon Collagen (Heat denatured)								
pH of solution at equilibrium	1.27	2.39	2.77	4.03	7.13	10.15	11.76	
Weight of residue (mg.)	751	1184	978	585	345	522	856	
Cattle Hide Collagen (Simple procedure)								
pH of solution at equilibrium	1.10	2.26	2.58	3.53	6.07	10.93	11.77	12.59
Weight of residue (mg.)	745	1640	1587	573	239	247	529	554

Pig Achilles Tendon Collagen

pH of solution at equilibrium	1.26	2.31	2.82	3.98	6.04	10.47	11.79	12.62
Weight of residue (mg.)	834	3898	3195	1794	198	280	835	1060

Pig Achilles Tendon Collagen (Prolonged treatment)

pH of solution at equilibrium		2.36			5.71			
Weight of residue (mg.)		3717			226			

Cattle Cornea Collagen (Prolonged treatment)

pH of solution at equilibrium	1.26	2.35	2.76	3.97	6.63	9.68	11.75	
Weight of residue (mg.)	892	1962	2152	668	326	484	1030	

Chicken Tarso-metatarsal Tendon Collagen

pH of solution at equilibrium	1.22	2.43	2.78	3.72	6.48	9.66	11.67	12.55
Weight of residue (mg.)	1288	7372	6394	1936	200	254	1336	1750

Rat Tail Tendon Collagen

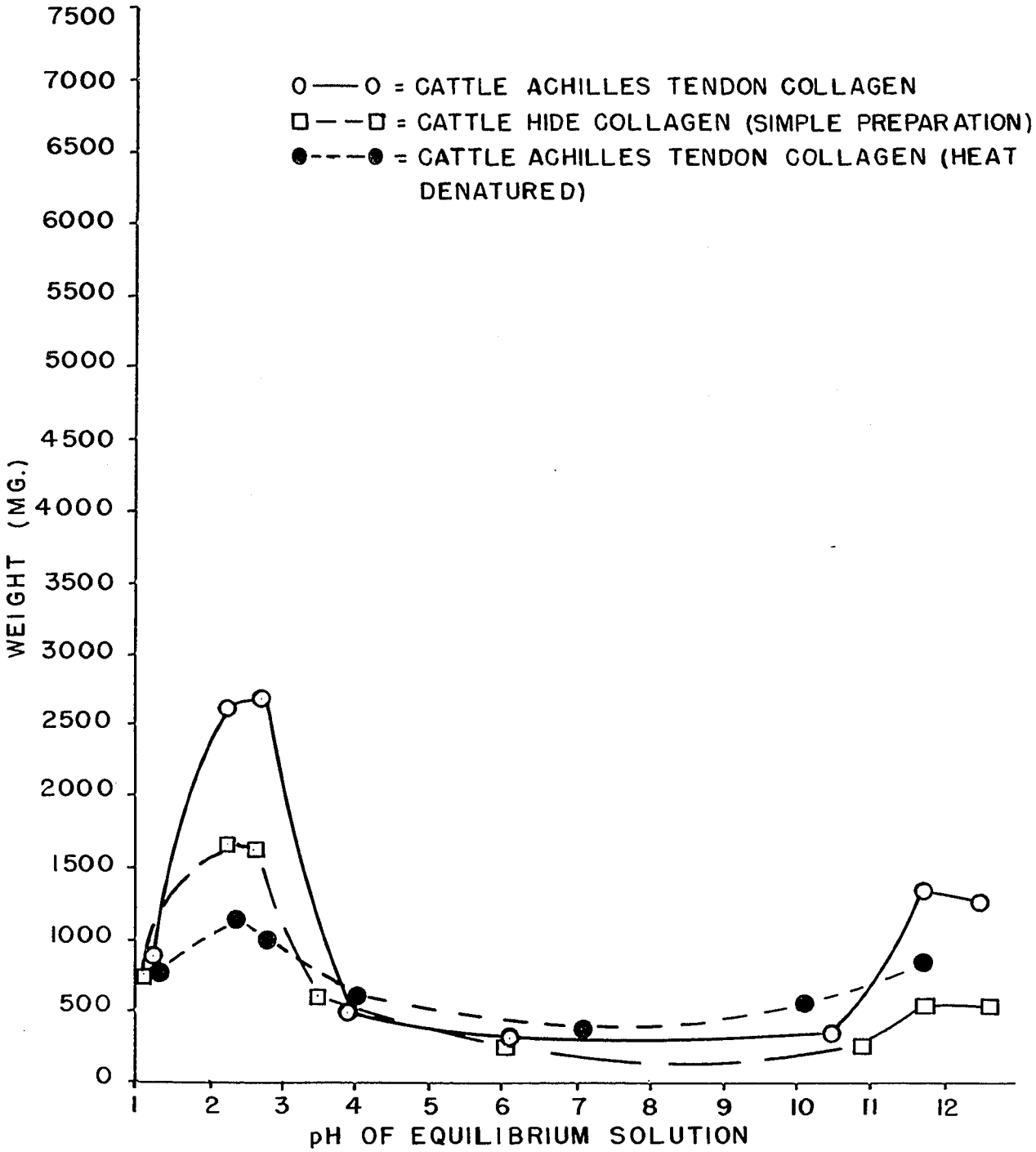
pH of solution at equilibrium	1.22	2.29	2.75	4.32	4.90	8.93	11.73	12.59
Weight of residue (mg.)	1740	- - - - Solubilized - - - - -			1167	218	2301	2431

Halibut Skin Collagen

Appreciable solubilization except at approximate pH's 7 and 11. At these pH's no noticeable swelling took place.

Graph IV. The Swelling of Collagens as a Function of pH.

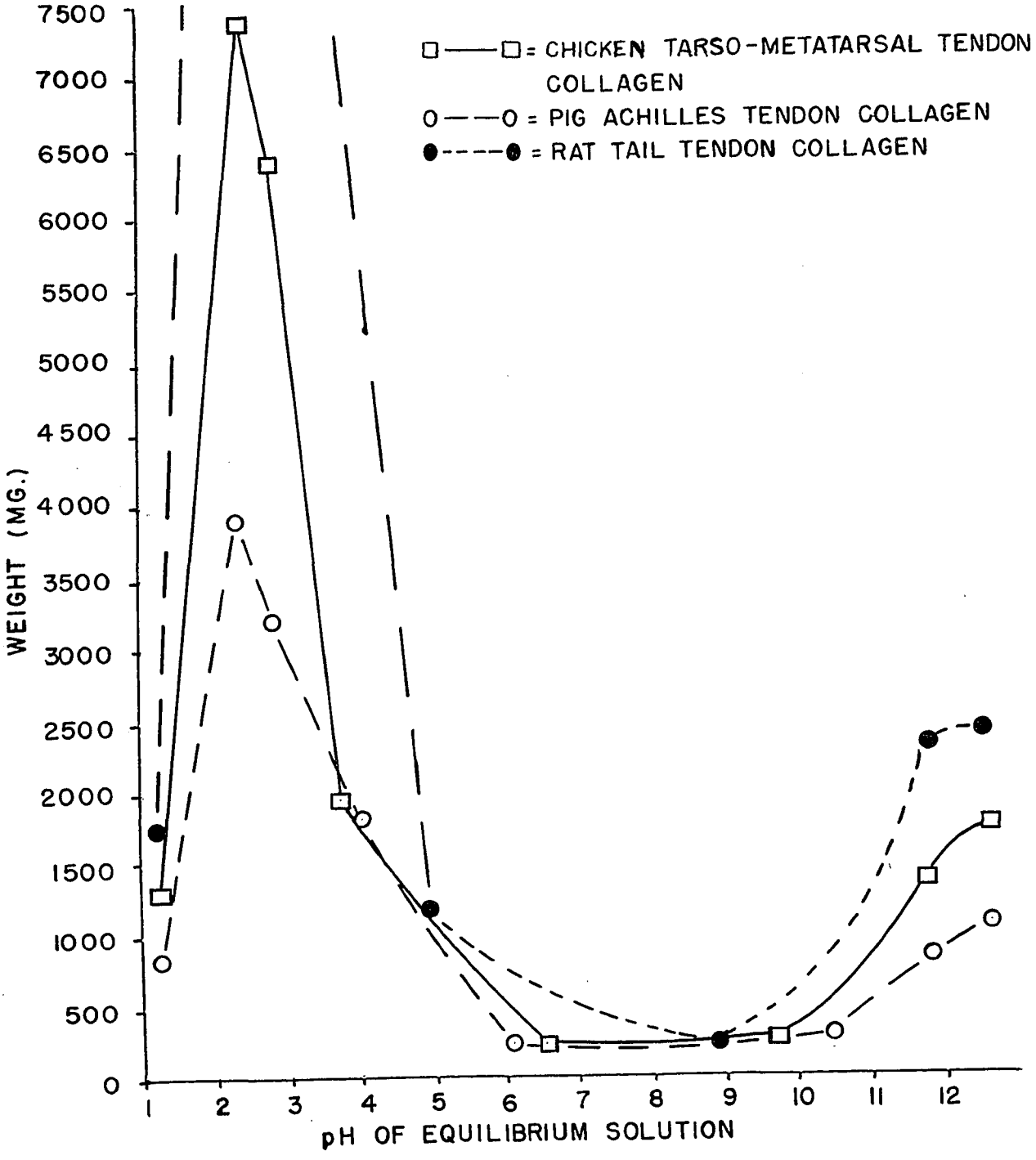
Collagens: Cattle achilles tendon collagen (native and denatured), cattle hide collagen (simple preparation).



Graph V. The Swelling of Collagens as a Function of pH.

Collagens: Pig achilles tendon collagen, rat tail tendon collagen, chicken tarso-metatarsal tendon collagens.

Between pH 1.2 and pH 4.9 the swelling curve for rat rail tendon collagen is hypothetical.



THE ACTION OF PROTEOLYTIC ENZYMES ON COLLAGEN

Native collagen is generally considered resistant to trypsin (4, 31). Pepsin attacks collagen (31, 48). Blow fly larva (49), the mold, A. oryzae (50), and a crayfish (51) are said to produce collagenases, proteases attacking collagen. Production of a collagenase by Cl. perfringens is reported by Maschmann (52), Macfarlane (53), and Oakley et al. (54). Jennison (55) made a survey of a qualitative nature of a number of microorganisms. He stated that Cl. perfringens had no observable effect on collagen though Cl. histolyticum was highly active. At the same time B. mycoides, B. mesentericus, Cl. sporogenes, Cl. bifermentans, and Cl. lentoputrescens demonstrated digestive action on collagen.

In previous studies little or no attention was given to the effect of source of the collagen on the susceptibility to enzymes. The term "collagenase" as used in the past has implied capacity for digestion of any collagen whatsoever. Another important consideration is the preparative procedures to which the collagen was subjected. Heating at mild temperatures in water results in denaturation and susceptibility to trypsin (4). In a number of studies of collagenase in the literature altered substrates have been used for detection, thereby giving rise to erroneous results.

In the present study a survey is made of the digestive action of six proteolytic enzymes on collagen from various sources prepared by procedures calculated not to alter the structure.

EXPERIMENTAL

Digestion of Hide Powder and Collagen by Enzymes

In order to demonstrate digestion of collagen by proteases the collagens were incubated with the enzymes in buffered solutions, and digestion was determined by weighing the residues. Collagen was prepared by the brief treatment described in the section on preparation of collagen. Cattle, pig, and rat tail tendon subjected to prolonged treatment was not significantly different from that prepared by the brief treatment in susceptibility to trypsin or Cl. perfringens enzymes. Kangaroo tail tendon was the air-dried whole tendon cut into cross sections of 2-3 mm. in length. Two samples of cattle hide collagen were used, one prepared according to a procedure utilizing $Ca(OH)_2$ and trypsin (15), and the second by brief treatment with cold NaCl solution.

For trypsin (Merck 1:250) and chymotrypsin (crystalline) digestion, test tubes (18 x 150 mm.) containing 100 mg. of substrate and 4.0 ml. of 0.05 M phosphate buffer, pH 7.2, were placed in a water bath at 37° C. for a half hour to insure thorough wetting and attainment of bath tem-

perature. The trypsin and chymotrypsin were added in 1.0 ml. of the same buffer solution at 37° C.

Digestion medium for pepsin was 10.0 ml. of N/100 HCl. In this case the substrate was suspended in 9.0 ml. of the HCl solution, and the pepsin in 1.0 ml. of the HCl solution was added.

Papain digestion was conducted as the trypsin except that the buffer solutions were M/100 with respect to KCN.

Cl. histolyticum and Cl. perfringens enzyme preparations were produced in culture filtrates on a medium previously described (56). Cl. perfringens enzyme preparations also were prepared by $(NH_4)_2SO_4$ precipitations. The digestions with bacterial filtrates were conducted similarly to those with trypsin except that 2.0 ml. phosphate buffer was used when 3.0 ml. of filtrate adjusted to pH 7.2 was added. No activation of the bacterial filtrates by KCN or cysteine occurred, as measured on gelatin and hide powder. The pH of 7.2 was chosen because it was the optimum pH for Cl. perfringens enzyme preparation and it allowed high activity for all enzymes except pepsin.

A layer of toluene was used as preservative in the tubes. Hide powder was subjected to the action of the enzyme preparations under the same conditions as a measure of the enzyme activity.

After periods of 1, 24, and 72 hours the residues were collected in a tared Gooch crucible, washed with water and then alcohol. The crucibles were dried to constant weight at 100-110° C. and weighed. A reasonably accurate measure of the digestive action of the enzyme preparations was thus obtained.

Post-enzymic Solubilization

It was earlier reported (1, 2) that trypsin pre-treatment of collagen from fish, cattle achilles tendon, and cattle hide considerably increased solubilization occurring at the shrinkage temperatures. The collagens, after incubation with trypsin, had been assiduously washed with water; so the possible effect of residual enzyme was discounted by the investigators. The solubilization was attributed to possible splitting of bonds by the tryptic digestion.

In the present study a number of different collagen preparations were treated with several enzymes. The washed residues were then heated in water to reveal enhanced ease of solubilization. Such an effect will be referred to as "post-enzymic solubilization."

The treatment of collagens with proteases was conducted as described in the preceding paragraphs. At the conclusion of the digestion period the residues were transferred to 15 ml. conical centrifuge tubes. The residues were centrifuged from the digestion medium and washing solutions. Over

a period of one hour the residues were suspended in five changes of 12 ml. portions of water at 25° C. Further washing with water did not decrease solubilization during the heat treatment. In the case of trypsin, practically all the trypsin was found free in the supernatant digestion mixture. M/10 NaCl, M/10 Na_2CO_3, M/10 NaOH and M/10 phosphate buffers of various pH were also used for washing. In these cases the residues were washed with four changes of 12 ml. portions of the solutions over a period of a half hour. Controls were washed with equal amounts of water for the same time. In washing out the M/10 NaOH, greater quantities of water were necessary to rapidly remove the NaOH and to reduce the swelling of the collagen which takes place in this concentration of NaOH. The removal of the NaOH was begun after 5 minutes standing with the NaOH. Controls washed with similar amounts of water gave the usual extensive solubilization upon heat treatment.

In certain cases, indicated in Table V, residues were suspended 5 minutes in solutions of sodium "Lorol" sulfate* which rinsed out with several portions of water. The total washing time was one hour.

All the washings were conducted at room temperature (25° C.).

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* A wetting agent supplied by E. I. DuPont de Nemours Co.

After the washings the residues were mixed in the centrifuge tubes with 5.0 ml. of water. The tubes were placed in a water bath at the designated temperature $\pm 1^{\circ}$ C. for 20 minutes. For most of the work 66° C. was chosen because it was $2\text{-}5^{\circ}$ C. higher than the temperature required to produce visible shrinkage of all collagens tested. This visible shrinkage is accompanied by an increased susceptibility to proteolytic action. In several instances after incubation with papain a 75° C. heat treatment was used because in those cases 75° C. treatment solubilized more effectively than did 66° C.

Heat treatment was also conducted in the presence of sodium "Lorol" sulfate and of H_2O_2. 5.0 ml. of solution containing the stated amount of inhibitor was added to the washed residues, stirred, allowed to stand 5 minutes, and then placed in the water bath for 20 minutes at the designated temperature.

After the heat treatments the residues were collected, washed, dried, and weighed as usual.

Heat and Urea Denaturation

The increase of susceptibility of collagen to trypsin caused by denaturation was determined in the follow- manner:

Heat denaturation was accomplished by heating

100 mg. of cattle achilles tendon collagen in 5.0 ml. of water at 65° C. for 20 minutes. The suspension had been maintained for an hour at room temperature (25° C.) to assure thorough wetting.

Urea denaturation was accomplished by suspension of 100 mg. of the collagen in 5.0 ml. of 4 M, 5 M, and 6 M urea at 37° C. for 24 hours.

After the denaturation procedures the supernatant was removed and the urea was thoroughly washed out.

To the residues was added 5.0 ml. of 0.05 M phosphate buffer pH 7.2 containing 1.0 mg. trypsin (1:250), or 2.0 mg. Cl. perfringens enzyme preparation, or 2.0 mg. papain. The tubes containing papain were also made 0.01 N with respect to KCN.

The tubes were incubated 24 hours at 37° C. Finally, the residues were collected in Gooch crucibles, washed successively with water and alcohol, dried to constant weight, and weighed.

RESULTS

Digestion of Collagens by Enzymes

It is obvious, as shown in Table VIII, that hide powder is highly susceptible to attack by all the proteases tested. Extensive and rapid digestion of all collagen samples was obtained only with Cl. histolyticum filtrates and with pepsin. Cl. perfringens enzyme preparations were 10-20 fold less active than equal quantities of Cl. histolyticum filtrates. It is interesting to note that collagen prepared by $Ca(OH)_2$ and trypsin treatment (15) is much more susceptible to digestion by all the enzymes than are the other collagen preparations. This may be due to modification of the collagen by the more drastic preparative procedures. This possibility is strengthened by the relative large extent of digestion of this collagen preparation occurring in the first hour of incubation. The small quantities of the other collagen preparations that are digested by trypsin, papain, and chymotrypsin may be contaminating proteins or collagen denatured either previously or during incubation. As stated earlier in the discussion of preparative procedures, the collagen preparations from tendons were shredded in a Waring blendor. The hide collagen samples were ground in a Wiley mill. Only the kangaroo tail tendon

was in relatively large pieces, consisting of the whole tendons in 2-5 mm. lengths. The effect of the relative surface exposed to enzyme action may be discounted because of: 1) the long incubation period, and 2) the large degree of solubilization produced by the "collagenases" regardless of particle size. Collagen preparations from chicken tendon, turtle subcutaneous membranes, cattle tail tendon, cattle chordae tendinae, cattle bone, and sheep tendon showed resistance to trypsin and the susceptibility to Cl. histolyticum filtrates typical of the other collagen preparations.

Post-Enzymic Solubilization

In Table IX are recorded data showing that all collagen samples tested demonstrated a greatly increased solubilization at 60-75° C. after incubation with several proteases and subsequent thorough water washing. The enzymes producing this effect were trypsin, crystalline trypsin, and papain. Enzymes not producing appreciable effects were crystalline chymotrypsin and Cl. perfringens enzyme preparations. Previous investigators had washed the collagen only with water after the trypsin pretreatment.

It is shown in Table X that M/10 NaCl, Na_2CO_3, NaOH or phosphate buffers in contrast to water diminished the solubilization effect. Solutions were increasingly effective as the pH increased or decreased. The period of

washing was arbitrarily chosen to demonstrate relative effectiveness of the various washing solutions. Prolonging the washing time augmented the effect of all solutions whereas distilled water continued to have no effect. It also may be noted that incubation of collagen with trypsin or papain for two weeks resulted in less solubilization upon heat treatment than did shorter incubation.

In Table XI is shown the effect of altering the temperature of heat treatment. Cattle achilles tendon collagen was incubated with trypsin and papain for 1-24 hours at 37° C. The residues were thoroughly washed with distilled water. Instead of heating only at 66° C., heat treatment at 70-100° C. was applied. Heat treatment at 80-100° C. greatly diminished the extent of solubilization after trypsin pretreatment. It will be noted that papain, which is more resistant to high temperatures and high concentrations of urea than trypsin, actually was responsible for an increased solubilization at 75-90° C. At higher temperatures (100° C.) inhibition finally occurred. Sodium "Lorol" sulfate was found by preliminary tests to inhibit completely tryptic digestion of hide powder. As shown in Table XII inhibition of the post-enzyme solubilization also was accomplished by use of sodium "Lorol" sulfate. Post-enzyme solubilization resulting from papain was inhibited by H_2O_2 although that resulting from incubation with trypsin was not inhibited by H_2O_2.

It appears that the reported increased ease of solubilization of collagen after trypsin pretreatment is an effect of quantities of enzyme which are difficulty removed from collagen by washing in water at room temperatures (25° C.)

Heat and Urea Denaturation

Table XIII summarizes the observations on denaturation. Heating collagen in water at temperatures 2-5° in excess of the shrinkage temperatures renders cattle achilles tendon collagen similarly susceptible to three different proteases tested. Urea produces similar effects. In addition collagen preparations from sheep tendon, pig tendon, chicken tendon, turtle subcutaneous membrane, cattle tail tendon, kangaroo tail tendon, cattle chordae tendinae, cattle hide, and cattle bone were denatured by similar heat treatment and became susceptible to tryptic attack.

Summary

1) The use of hide powder as a substrate in the demonstration of collagenase activity is not valid.

2) Collagens from several sources, prepared by methods calculated not to alter properties, are resistant to the action of trypsin, chymotrypsin, and papain. The

collagens are readily attacked (solubilized by the proteolytic enzyme(s) of Cl. histolyticum and Cl. perfringens and by pepsin.

3) The reported increased solubilization of collagens in water at the shrinkage temperature (68-70°) after incubation with enzymes can be attributed to residual enzyme.

4) Denaturation of collagen by heat and urea produces a general susceptibility to proteolytic enzymes.

Suggestions for Future Work

A more detailed investigation of enzyme action on collagen would be of interest. Among the possibilities for profitable study are:

1) A further survey of enzymes for detection of collagenase activity.

2) Determination of properties and preparation of these enzymes, and the conditions for maximum action of collagens.

3) Characterization of the effects of collagenases on collagens; this would include determination of pre-solubilization effects, if any, and characterization of intermediary and end-products of digestion.

4) These enzymes once characterized might be of importance in (a) preparation of connective tissue proteins, (b) understanding of biological systems involving collagen,

(c) clinical chemistry, and (d) commercial utilization of collagen derivatives.

TABLE VIII

SOLUBILIZATION OF COLLAGENS BY ENZYMES*

Incubation Time	ENZYME							
	Trypsin	Cryst. Trypsin	Papain	Cryst. Chymotrypsin	Cl. perfringens pptd. enzyme	Cl. perfringens filtrates	Cl. histolyticum filtrates	Pepsin
Hours	%	%	%	%	%	%	%	%
Hide Powder								
1	59.4	60.3	53.8	57.7	20.6	22.5	50.4	7.7
24								88.5
Cattle Hide Collagen (Prepd. with $Ca(OH)_2$ and trypsin)								
1	19.0	10.8	14.1	17.4	9.8		21.8	0.0
24	28.2	15.2	21.3		27.4		74.9	95.0
72	34.4	22.2	22.0		49.8		95.8	
Cattle Achilles Tendon								
1	5.4	2.3	3.3	1.4	0.0	0.0	2.0	0.0
24	8.2	1.8	3.9	4.3	12.4	18.9	51.4	94.7
72	4.9	6.8	6.6	5.6	22.1	47.2	94.7	
Pig Achilles Tendon								
1	3.8					1.8		
24	5.7					21.4	97.4	95.7
72	5.0					63.1		
Kangaroo Tail Tendon								
1		2.4	1.3	0.0	2.9 **	0.0	1.3	
24	4.9	1.2	2.2	3.3	6.9 **	15.2	23.8	80.8
72	7.9	4.4	5.4	4.4	18.4 **	29.5	49.8	

1		2.4	1.3	0.0	2.9 **	0.0	1.3	
24	4.9	1.2	2.2	3.3	6.9 **	15.2	23.8	80.8
72	7.9	4.4	5.4	4.4	18.4 **	29.5	49.8	

Rat Tail Tendon

1						1.1		
24	4.9				36.9 **	43.6	91.8	
72	7.9				68.3 **	87.3		

* Enzymes used in the following quantities:

Trypsin (Merck 1:250)	1.0 mg.
Chymotrypsin (crystalline). . . .	0.2 mg.
Trypsin (crystalline)	0.1 mg.
Cl. perfringens filtrates	3.0 ml.
Cl. histolyticum filtrates . . .	0.2 ml.
Pepsin (U.S.P.)	1.0 mg.
Papain (KCN activates).	1.0 mg.
Cl. perfringens pptd.enzyme . . .	2.0 mg.

Temperature of incubation 37° C. ± 0.5°.

** 5.0 mg.

TABLE IX

POST-ENZYMIC SOLUBILIZATION OF COLLAGENS

Enzyme	Quantity of Enzyme (mg./tube)	Collagen Source	Amount of Substrate Solubilized by Heat %	Amount of Substrate Solubilized by Enzyme %	Amount of Substrate Solubilized by H + E %
Trypsin (Merck 1:250)	0	Cattle Achilles Tendon	0-0.5	---	---
	0.05			2.5	54
	0.5			4.0	87
	1.0			5.1	88
	2.0			4.4	91
	0	Pig Achilles Tendon	16-20	---	--
	0.05			2.0	71
	0.1			2.0	89
	0.5			2.0	97
	0	Sheep Achilles Tendon	2.3	---	--
	0.1			2.0	46
	0.5			3.6	95
	0	Chicken Tarso metatarsal Tendon	21.4	---	--
	0.05				48
	0.1				70
	0.5				97
	0	Kangaroo Tail Tendon	0	---	--
	1.0			4.0	14.2
	0	Cattle Hide (Prepd. by brief treatment)	9.4	---	--
	0.05				16
	0.1				39
	0.5			5.7	89
	0.05 (90 min.)	Hide Powder		41.7	
	0.15 (30 min.)			32.7	

	0.05 (90 min.) 0.15 (30 min.)	Hide Powder		41.7 32.7	
Crystalline Trypsin	0 0.1 0.5 1.0	Cattle Achilles Tendon	0-0.5	--- 0 0 1.1	-- 45 89 88
Crystalline Chymotrypsin	0 1.0 1.0 (24 hrs.)	Cattle Achilles Tendon	0-0.5	--- 0 4.1	-- 6.1 2.3
	0 1.0 1.0 (24 hrs.)	Pig Achilles Tendon	16-20	--- 2.0 5.0	-- 0 6.4
Papain	0 2.0 2.0 (24 hrs.)	Cattle Achilles Tendon	0-0.5	--- 2.9 4.0	-- 50-90 44
	0 (24 hrs.) 2.0 (24 hrs.) 2.0 (24 hrs.)		0-0.5*	---	-- 94* 94*
	0 (24 hrs.) 2.0 (24 hrs.)	Pig Achilles Tendon	26.7	---	-- 92
Cl. perfringens Powder #1412	0 10.0 10.0 (24 hrs.)	Cattle Achilles Tendon	0-0.5	--- 0 16.7	-- 6.5 12.5

* Heat treatment was for 20 minutes in a 75° C. water bath.
The time of incubation unless otherwise noted was one hour at 37° C. ± 0.5°.

TABLE X

POST-ENZYMIC SOLUBILIZATION OF COLLAGENS AFTER WASHING WITH VARIOUS SOLUTIONS

Enzymes	Collagen Source	Washing Solution	Post-Enzymic Solubilization %
Trypsin** (Merck 1:250) (1 mg./tube)	Cattle Achilles Tendon	M/10 NaH_2PO_4	13.8
		M/10 phosphate buffer, pH 6.0	31.2
		M/10 phosphate buffer, pH 6.5	50.0
		Distilled water	94.0
		M/10 NaCl	56.0
		M/10 phosphate buffer, pH 7.2	47.0
		M/10 phosphate buffer, pH 8.0	13.0
		M/10 Na_2HPO_4	16.0
		M/10 Na_2CO_3	9.0
		M/10 NaOH	8.0
Incubation time 24 hours		Distilled water	94.0
24 hours		M/10 NaOH	9.0
2 weeks		Distilled water	15.0
	Pig Achilles Tendon	M/10 NaH_2PO_4	79.0
		Distilled water	93.0
		M/10 phosphate buffer, pH 7.2	51.0

TABLE X (Contd.)

Enzyme	Collagen Source	Washing Solution	Post-Enzymic Solubilization %
	Pig Achilles Tendon	M/10 Na_2HPO_4	29.0
		M/10 NaOH	12.0
	Cattle Hide	Distilled water	86.0
		M/10 NaOH	14.0
Papain*** (2 mg./tube)	Cattle Achilles Tendon	M/10 phosphate buffer, pH 6.0	30.0
		Distilled water	56.0
		M/10 phosphate buffer, pH 7.2	44.0
		M/10 Na_2HPO_4	49.0
		M/10 NaOH	5.0
		Distilled water	94.0*
		M/10 NaOH	15.0*
Incubation time 2 weeks		Distilled water	50.0*
	Pig Achilles Tendon	Distilled water	95.0*
		M/10 NaOH	10.0*

* Heat treatment was 20 minutes in water bath at 75° C.
** Incubation for 1 hour unless otherwise noted.
*** Incubation for 24 hours unless otherwise noted.

TABLE XI

POST-ENZYMIC SOLUBILIZATION OF CATTLE ACHILLES TENDON BY HEAT TREATMENT AT VARIOUS TEMPERATURES

Enzyme	Incubation Time*	Temperature of Heat Treatment (° C.) 66	70	75	80	85	95	100
	hours	% solubilized						
Trypsin (Merck 1:250)	1.0	74	74	62	28	12		12
	24.0	94	14	16		12		
	1.0**	86				20		
Papain	2.0	72		94		94	94	27
	24.0	53	81	94	94	94		30

* Incubation at 37° C. ± 0.5°
** Cattle hide collagen (mild preparation).

TABLE XII

POST-ENZYMIC SOLUBILIZATION OF CATTLE ACHILLES TENDON COLLAGEN AFTER TREATMENT WITH SPECIFIC ENZYME INHIBITORS

Enzyme	Incubation Time	Treatment after Water Washing	Post-Enzymic Solubilization
	hours		%
Trypsin (Merck 1:250) (1 mg./ tube)	1.0	66° - heat treatment* in distilled water	94
	1.0	5 minutes in 0.2% Na "Lorol" sulfate - Washed out, then 66° heat treatment	57
	1.0	5 minutes in 1.0% Na "Lorol" sulfate - washed out, then 66° heat treatment	9
	1.0	5 minutes in 2.0% Na "Lorol" sulfate - washed out, then 66° heat treatment	5
	24.0	5 minutes in 2.0% Na "Lorol" sulfate - washed out, then 66° heat treatment	5
	1.0	66° heat treatment in presence of 0.04% Na "Lorol" sulfate	66
	1.0	66° heat treatment in presence of 0.2% Na "Lorol" sulfate	9
	1.0	66° heat treatment in 0.1% H_2O_2	94
Papain (2 mg./ tube)	2.0	75° heat treatment in distilled water	90
	2.0	75° heat treatment in 0.0001% H_2O_2	90

TABLE XII (Contd.)

Enzyme	Incubation Time	Treatment after Water Washing	Post-Enzymic Solubilization
	hours		%
Papain (2 mg./ tube)	2.0	75° heat treatment in 0.001% H_2O_2	73
	2.0	75° heat treatment in 0.01% H_2O_2	5
	2.0	75° heat treatment in 0.1% H_2O_2	3

All heat treatments were for 20 minute periods in water bath at designated temperatures.

TABLE XIII

DIGESTION OF COLLAGEN BY PROTEOLYTIC ENZYMES AFTER HEAT TREATMENT AND INCUBATION WITH UREA

Enzyme	Pre-treatment of Collagen	Amount of Solubilization
		%
Trypsin*	None	2.5
	20 minutes at 65° C.	68.2
Papain**	None	0
	20 minutes at 65° C.	68.2
Cl.perfringens pptd.enzyme***	None	6.2
	20 minutes at 65° C.	71.8
Trypsin*	Distd.water 24 hrs. at 37° C.	3.3
	4 M urea - 24 hrs. at 37° C.	0
	5 M urea - " " "	35.9
	6 M urea - " " "	59.0
Papain**	Distd.water 24 hrs. at 37° C.	1.4
	4 M urea	6.6
	5 M urea	31.6
	6 M urea	58.8
Cl.perfringens pptd.enzyme***	Distd.water 24 hrs. at 37° C.	16.4
	4 M urea - 24 hrs. at 37° C.	10.5
	5 M urea - " " "	43.4
	6 M urea - " " "	70.0

* Trypsin at 1.0 mg. per tube.
** Papain (KCN activated) at 2.0 mg. per tube
*** Cl. perfringens pptd. enzyme at 2.0 mg. per tube
Incubation with enzyme 24 hours at 37° C. ± 0.5°.

ACTION OF PROTEOLYTIC ENZYMES ON ELASTIN

It is generally accepted that trypsin slowly attacks elastin (28, 57). It is also stated that certain microorganisms may attack elastin (58). Elastin has been reported to be resistant to Cl. perfringens enzymes (52). Pepsin, too, is said to attack elastin slowly (28, 57, 25). A blow-fly larva is said to attack elastin (49). The few references indicate that the study of enzyme action on elastin is a neglected subject.

In the present paper a survey is made of the action of six proteolytic enzymes on elastin.

Experimental

"Native" elastin was prepared from cattle ligamentum nuchae by extractive procedures described except that no hot solvents were used. (See preparation of elastin.) This sample was determined by the method of Lowry (23) to contain approximately 80% elastin and 20% collagen. Cattle ligamentum nuchae elastin samples also were prepared by the procedures involving heating 24 hours at 100° C. in water and for 24 hours at 100° C. in 40% urea. These latter two samples were assumed to be free of collagen and to contain 95-100% elastin.

The digestion procedures and the measure of solubilization were conducted in the same manner as in the

collagen digestion studies (see page 46).

RESULTS

From the data contained in Table XIV it is apparent that, in contrast to collagen, elastin was appreciably attacked by trypsin and papain whereas it was relatively resistant to pepsin, Cl. perfringens enzymes, and Cl. histolyticum enzymes. Elastin also was resistant to chymotrypsin. The results obtained on "native" elastin include the effects from the collagen content of this preparation. It will be observed that the samples heated in water were more rapidly solubilized than the native elastin. This effect was even greater with samples heated in 40% urea. It has been reported (59, 60) that heating in water brings about a gradual conversion of elastin to another form differing somewhat in elastic properties.

These experiments suggest that further knowledge of enzyme specificity and protein structure may be gained by extension of such studies. The effects of Cl. perfringens enzymes in pulping of muscle in gas gangrene may be entirely due to attack on constituents other than elastin. In the digestive system the specificities of two of the principal proteases may be complementary with respect to a number of common substrates - collagen being readily attacked by pepsin whereas elastin is attacked by trypsin. A knowledge of enzyme action on elastin and collagen may contribute to

the development of purification methods for these proteins.

Suggestions for Future Work

A more detailed investigation of enzyme action on elastin would be of interest. Among the suggestions for profitable study are:

1) A further survey of enzymes for detection of proteolytic action on elastins.

2) Determination of properties and preparation of these enzymes and the conditions for maximum action on elastins.

3) Characterization of the effects of proteases on elastins; this would include determination of pre-solubilization effects, if any, and characterization of intermediary products and end-products of digestion.

4) These enzymes once characterized might be of importance in (a) preparation of connective tissue proteins, (b) understanding of biological systems involving elastin, and (c) clinical chemistry.

TABLE XIV

PER CENT SOLUBILIZATION OF ELASTIN BY PROTEOLYTIC ENZYMES*

Incubation Time hours	Trypsin	Cryst. Chymotrypsin	Papain	Pepsin	Cl. perfringens Filtrate	Cl. histolyticum Filtrate
			Hide Powder			
1	67.7	55.6	65.6	8.9	25.2	36.6**
24	--	--	--	68.0	--	--
			Native Elastin			
1	3.6	0	4.5	2.0	0	0
24	36.5	3.2	31.8	8.0	4.3	13.3
72	76.1	3.2	54.2	32.3	12.1	16.1
			Elastin, 24 hours in water at 100° C.			
24	65.2	6.1	36.2	26.2	--	--
			Elastin, 24 hours in 40% urea at 100° C.			
24	83.3	--	53.6	31.6	--	--

* Quantity of enzyme preparation per tube was as follows: trypsin, 1.0 mg.; crystalline chymotrypsin, 0.2 mg.; papain, 2.0 mg.; pepsin, 1.0 mg.; Cl. perfringens filtrate, 3.0 ml.; Cl. histolyticum filtrate, 0.2 ml.

** 34.2% of cattle achilles tendon collagen was solubilized by 0.02 ml. Cl. histolyticum filtrate in 24.0 hours.

AMINO ACID COMPOSITION OF GELATINS, COLLAGENS, AND ELASTINS

Introduction

Collagen. The amino acid composition of collagen previously has been largely determined by analyses of its derivative, gelatin. This is probably due to the ready availability of gelatin and the assumption that its composition truly represents that of its precursor. However, commercial gelatins are frequently of doubtful origin and content. Preparative procedures for gelatin may partially destroy amino acids such as arginine and lysine (61). Collagen itself has been assayed for a number of amino acids. Usually the collagen source was cattle hide.

In general, only a few constituents of a given sample have been assayed. In addition, assay procedures available have been crude. In consequence of these several factors, reported values for the different amino acids have been widely variant; also collagens from only a few sources have been investigated to any extent.

In the present study an almost complete amino acid assay has been made of four gelatins and thirteen collagen preparations from different animals and tissues.

Elastin. Information regarding the composition of elastin is exceedingly scanty. The sole source of elastin for all previous amino acid assay has been cattle ligamentum nuchae.

In the present study elastin has been prepared from the ligamentum nuchae of cattle and sheep, and from the the aortae of cattle, sheep, and pigs. The assays conducted on collagen and gelatin were also conducted on the elastins.

Experimental

Collagens and elastins were prepared by the prolonged extractive treatments to remove impurities. Fish scale gelatin was prepared by autoclaving miscellaneous fish scales in water two hours at 15 pounds pressure.

For the microbiological assays, protein hydrolysates were prepared as follows: About 0.5 g. of protein was dried overnight at 100-110° C., and then weighed in glass-stoppered weighing bottles. The sample was transferred to 17 x 150 mm. pyrex test tubes. After adding 5.0 ml. of 2 N HCl, the tube was sealed by fusion. The sample was then autoclaved 10 hours at 15 pounds. The hydrolysate was neutralized, filtered, and brought to 50.0 ml. volume. This procedure is similar to that of other investigators using microbiological assays (62). There apparently was no appreciable destruction of any of the amino acids for which analyses were made.

Cl. perfringens BP6K was used according to the method of Boyd, Logan, and Tytell (63) for the determination of twelve amino acids: isoleucine, leucine, valine, threonine, phenylalanine, tyrosine, tryptophan, methionine, cystine, arginine,

histidine, and glutamic acid. The synthetic medium used permitted dense growth in 16 hours at 45° C. At the same time, in the absence of the amino acid assayed for, growth was invariably absent or negligible. Growth was measured with a sensitive turbidimeter (64, 63). In general, the middle half of the growth curve was used. Assays at different levels in this range yielded equivalent values. No hydrolysate had a stimulatory effect on growth other than that accountable for by the amino acid being determined.

Leuconostoc mesenteroides P-60 was used for the assay of (1) lysine, (2) aspartic acid, (3) glycine, (4) proline. Several different assay procedures were used (65, 66, 67, 68). The assay values obtained were the same regardless of the different media. However, the medium of Steele et al. (66) yielded greatest maximal growth as measured turbidimetrically. Values obtained upon a medium allowing maximal growth might be less affected by unknown stimulatory or inhibitory factors in the hydrolysates. Growth for amino acid assay was measured turbidimetrically. Assay values were constant after 20-72 hours growth at 34-37° C. as measured turbidimetrically. Acid production was equal to that obtained by the original investigators.

Leuconostoc citrovorum 8081 was used for the assay of alanine according to the procedure of Sauberlich and Bauman (69). The medium containing the pyridoxine vitamins

was chosen. Growth was measured turbidimetrically after 18 hours growth.

Leuconostoc mesenteroides and Leuconostoc citrovorum stock cultures were carried as stab cultures in yeast extract-glucose-agar (1% glucose, 1% yeast extract, 1.5% agar) and the inoculum for assays was grown in a medium containing Bacto-peptone, Bacto-yeast extract, acetate, glucose, and inorganic salts, as described by McMahan and Snell (62).

Serine was determined by the colorimetric method of Boyd and Logan (70). The values obtained represent both serine and hydroxylysine.

A colorimetric method of Guest and MacFarlane (71) for hydroxyproline was applied to the hydrolysates, but results were unsatisfactory. No microbiological or chemical method is entirely satisfactory for this amino acid. Further work is planned to contribute to the solution of this problem.

Standards for the amino acids were for the most part crystalline products of Merck and Co. Optical rotation and nitrogen content, indicating high purity of these preparations, were declared by the company. Use of these amino acids in microbiological assay media revealed negligible contamination by other amino acids. In one case, a known exception was discovered. Merck dl-alanine contained glycine in sufficient quantity to interfere with the assay for glycine. A

single recrystallization removed the glycine. This recrystallized dl-alanine was used as standard. All standards were stored over P_2O_5 in a desiccator.

The expression "%" was used to mean "g. amino acid/100 g. moisture-free protein" throughout the discussion of amino acid content.

Results

The amino acid content of the majority of various preparations agreed closely with data obtained with reliable methods used by other workers. Apparently the amino acid composition of collagens from different animals and tissues are closely alike. In lower vertebrates such as reptiles and fish as many as three amino acids, serine, threonine, and methionine, appeared to vary widely from the values common for collagens from mammals and birds. In the instances in which these amino acids varied they occurred in 50-100% higher amounts than in the usual mammalian or avian collagen. It is possible that these variations represent impurities.

In general, elastin from the ligamentum nuchae and the aorta of cattle, sheep, and pigs contained the same amino acid composition. However, pig aorta elastin contained significantly less isoleucine and more tyrosine and glutamic acid.

Table XV is a compilation of the amino acid composition determined for the gelatins, collagens, and elastins.

In later pages is given a more detailed account of the assays.

Table XVI provides a calculation of the amino acid residue* content and per cent of the protein by weight accounted for by the present study of composition. It will be observed that values for amino acids of elastin were usually equal or greater than previously reported values; yet the total residues are only about 90% of the dry weight of the protein. The nitrogen of cattle aorta elastin accounted for by the assays was 93.3% as compared with 91.1% of dry weight accounted for. It can be supposed that non-basic amino acids are responsible for the remaining nitrogen. Several per cent higher results of glycine and proline could account for the remainder of the elastin; assay of these amino acids are least reliable among the amino acid determinations.

Suggestions for Future Work

Future work of value perhaps would consist of:

1) Development and application of improved assay procedures for hydroxyproline, hydroxylysine, and serine (by microbiological assay).

2) An accurate and detailed study of the occurrence of serine, threonine, hydroxylysine, methionine, and hydroxy-

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* An amino acid residue is that portion of an amino acid remaining after forming two peptide linkages as in a polypeptide chain.

proline in purified preparations of collagen from lower organisms.

3) An extension of complete analyses to a wider and more representative group of organisms.

4) Correlation, if possible, of the assay results with the physical and biochemical properties of collagen.

5) Similar studies of elastin from different sources. In this study particular attention might be given to isoleucine, glutamic acid, and tyrosine.

At this time there is planned an extension of composition studies to other structural proteins.

Collagens	Glycine	Alanine	Iso-leucine	Leucine	Valine	Se
Cattle Hide (Prepd.with $Ca(OH)_2$ and trypsin)	27.5	8.8	2.2	3.3	2.5	
Cattle Hide (Treated with NaCl solution)	29.3	10.0	2.1	3.2	2.7	
Cattle Bone	23.3	--	2.1	3.4	2.7	
Cattle Achilles Tendon	24.8	8.7	2.1	3.5	2.8	
Pig Achilles Tendon	26.6	8.7	1.7	3.4	2.7	
Sheep Achilles Tendon	25.1	9.1	1.7	3.4	3.0	
Rhesus Monkey Achilles Tendon	24.4	8.7	--	--	--	
Chicken Tarso-metatarsal Tendon	26.2	9.3	2.0	3.4	2.4	
Cattle Tail Tendon	28.0	8.7	1.9	3.6	3.2	
Rat Tail Tendon	28.9	9.7	1.9	3.2	2.9	
Kangaroo Tail Tendon	26.4	8.7	1.5	3.0	2.7	
Turtle Subcutaneous Membrane	26.9	9.1	1.8	3.5	2.3	
Fish Skin	29.6	9.2	1.7	3.7	2.5	

TABLE XV (Part 1)

THE AMINO ACID COMPOSITION OF GELATINS, COLLAGENS, AND ELASTINS

Valine	Serine	Threo-nine	Proline	Phenyl-alanine	Tyrosine	Trypto-phan	Methio-nine	Cys
2.5	2.8	2.2	15.8	2.3	0.89	--	0.89	0.
2.7	2.8	2.2	16.5	2.4	1.00	--	0.94	0.
2.7	3.1	2.3	--	2.6	0.98	<0.01	0.72	0.
2.8	3.2	2.3	13.3	2.4	0.91	<0.01	0.84	0.
2.7	3.1	2.3	14.3	2.4	0.96	<0.01	0.89	0.
3.0	3.0	2.5	14.1	2.3	0.89	<0.01	0.86	0.
--	--	2.3	--	2.4	1.02	--	0.98	--
2.4	2.8	2.5	15.7	2.6	0.90	< 0.01	1.16	0.
3.2	3.2	2.3	14.9	2.4	0.98	--	0.89	--
2.9	3.2	2.5	15.5	2.6	1.06	--	0.96	0.
2.7	3.1	2.3	--	2.5	0.93	--	0.91	0.
2.3	--	3.4	15.6	--	0.97	--	0.68	--
2.5	5.9	3.5	13.7	2.4	0.86	--	2.3	0.

ELASTINS

o-	Methio-nine	Cystine	Histi-dine	Arginine	Lysine	Aspartic Acid	Glutamic Acid
	0.89	0.04	0.62	8.7	5.3	7.0	11.4
	0.94	0.02	0.74	8.6	5.5	7.5	10.8
	0.72	0.11	0.64	9.0	5.5	--	11.2
	0.84	0.11	0.75	9.0	4.9	6.7	11.9
	0.89	0.10	0.66	9.0	5.2	6.7	11.9
	0.86	0.10	0.67	8.9	5.3	6.7	11.5
	0.98	--	--	8.6	5.1	7.0	--
	1.16	0.12	0.68	8.9	5.5	6.7	12.2
	0.89	--	0.80	8.7	5.3	6.9	11.7
	0.96	0.08	0.56	8.9	5.7	6.9	11.9
	0.91	0.10	--	8.6	5.5	7.0	11.6
	0.68	--	0.68	9.0	5.1	5.2	11.9
	2.3	0.12	0.94	9.5	5.6	7.1	11.5

	Glycine	Alanine	Iso-leucine	Leucine	Valine	Serir
GELATINS						
Difco	25.7	8.6	1.5	3.1	2.8	3.2
Calf Skin (Eastman, purified)	26.9	8.7	1.9	3.1	2.6	2.9
Pig Skin " "	30.5	9.2	1.5	3.2	2.7	2.9
Fish Scale	27.4	--	1.2	2.5	2.2	3.5
ELASTINS						
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	--	18.4	4.3	8.4	18.4	1.0
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	29.9	18.9	4.0	8.7	17.4	0.8
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	--	--	3.8	8.5	15.5	
Sheep aorta (40 hrs. in 40% urea at 100° C.)	27.9	20.0	3.6	8.1	18.4	
Cattle aorta (40 hrs. in 40% urea at 100° C.)	30.2	19.3	3.9	8.7	17.6	0.8
Pig aorta (40 hrs. in 40% urea at 100° C.)	28.8	20.1	2.7	7.9	16.5	0.8

TABLE XV (Part 2)

THE AMINO ACID COMPOSITION OF GELATINS, COLLAGENS, AND ELASTINS

Serine	Threonine	Proline	Phenylalanine	Tyrosine	Tryptophan	Methionine	Cystine
3.2	2.0	16.3	2.3	0.91	--	0.92	0.09
2.9	2.2	14.0	1.9	0.14	--	0.85	0.05
2.9	2.2	16.3	2.1	0.69	--	0.80	0.09
3.5	3.3	14.1	2.2	0.72	--	2.2	0.17
1.00	1.15	--	5.7	1.85	<0.01	0.03	0.25
0.82	0.96	17.0	5.0	1.61	< 0.01	0.03	0.15
	1.6	--	5.4	2.36	--	0.12	--
	1.16	15.8	5.0	2.10	--	0.00	--
0.80	1.01	16.8	5.1	1.63	--	0.00	--
0.82	1.31	15.4	5.4	2.91	--	0.00	--

ASTINS

Methionine	Cystine	Histidine	Arginine	Lysine	Aspartic Acid	Glutamic Acid
0.92	0.09	0.85	8.3	5.2	6.4	11.5
0.85	0.05	0.63	6.4	5.2	6.9	12.1
0.80	0.09	0.67	8.8	5.1	6.3	11.7
2.2	0.17	1.01	8.9	4.9	6.5	11.4
0.03	0.25	0.05	1.05	0.40	—	2.2
0.03	0.15	0.07	0.89	0.39	0.63	2.1
0.12	--	0.21	--	0.42	--	1.9
0.00	--	--	0.90	0.37	0.61	--
0.00	--	--	0.87	0.47	0.64	2.1
0.00	--	--	0.93	0.51	0.44	2.8

THE AMIN

	Glycine	Alanine	Iso-leucine	Leucine	Valine	Seri
GELATINS						
Difco	19.5	6.9	1.3	2.7	2.4	2.
Calk Skin (Eastman purified	20.4	6.9	1.6	2.7	2.2	2.
COLLAGENS						
Cattle Hide (prepd. with $Ca(OH)_2$ + trypsin)	20.9	7.0	1.9	2.8	2.1	2.
Cattle Hide (prepd. with NaCl soln.)	22.3	8.0	1.8	2.8	2.3	2.
Cattle Achilles Tendon	18.9	6.9	1.8	3.0	2.4	2.
Pig Achilles Tendon	20.2	6.9	1.5	2.9	2.3	2.
Sheep Achilles Tendon	19.1	7.3	1.5	2.9	2.5	2.
Chicken Tarso-metatarsal Tendon	19.9	7.4	1.7	2.9	2.0	2.
Cattle Tail Tendon	21.3	6.9	1.6	2.8**	2.7	2.
Rat Tail Tendon	22.0	7.7	1.6	2.8	2.5	2.
Fish Skin	22.5	7.3	1.5	3.2	2.1	4.
ELASTINS (Treated 40 hrs. in 40% urea at 100° C.)						
Cattle Ligamentum nuchae	22.7	15.1	3.5	7.5	14.7	0.
Sheep Aorta	21.2	16.0	3.1	7.0	15.6	0.
Cattle Aorta	23.0	15.4	3.3	7.5	14.9	0.
Pig Aorta	21.7	16.1	2.3	6.8	14.0	0.

* Calculated from "best" values in literature.
** Assumed.

TABLE XVI

THE AMINO ACID RESIDUE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Valine	Serine	Threo-nine	Proline	Phenyl-alanine	Hydroxy-proline*	Tyrosine	Methio-nine	Cy
2.4	2.7	1.7	13.8	2.1	12.6	0.7	0.8	
2.2	2.4	1.8	11.8	1.7	12.6	0.1	0.8	
2.1	2.3	1.8	13.3	2.1	12.6	0.7	0.8	
2.3	2.3	1.8	13.9	2.1	12.6	0.8	0.8	
2.4	2.7	1.9	11.2	2.1	12.6	0.7	0.7	
2.3	2.6	1.9	12.1	2.1	12.6	0.8	0.8	
2.5	2.5	2.1	11.9	2.1	12.6	0.7	0.8	
2.0	2.4	2.1	13.2	2.3	12.6	0.7	1.0	
2.7	2.7	1.9	12.6	2.1	12.6	0.8	0.8	
2.5	2.7	2.1	13.1	2.3	12.6	0.9	0.9	
2.1	4.9	2.9	11.6	2.1	12.6	0.7	0.7	
14.7	0.7	0.8	14.3	4.5	1.7	1.3	0.0	
15.6	0.7**	1.0	13.3	4.5	1.7	1.7	0.0	
14.9	0.7	0.9	14.2	4.5	1.7	1.3	0.0	
14.0	0.7	1.1	13.0	4.8	1.7	2.3	0.0	

ELASTINS

ine	Methio-nine	Cystine	Histi-dine	Arginine	Lysine	Hydroxy-lysine*	Aspartic Acid	Glutar Acic
	0.8	0.1	0.7	7.4	4.6	0.9	5.5	10.1
	0.8	--	0.5	5.7	4.6	0.9	6.0	10.6
	0.8	--	0.5	7.8	4.6	0.9	6.1	10.0
	0.8	--	0.6	7.7	4.8	0.9	6.5	9.5
	0.7	0.1	0.6	8.1	4.3	0.9	5.8	10.4
	0.8	0.1	0.6	8.1	4.6	0.9	5.8	10.4
	0.8	0.1	0.6	8.0	4.6	0.9	5.8	10.1
	1.0	0.1	0.6	8.0	4.8	0.9	5.8	10.7
	0.8	0.1	0.7	7.8	4.6	0.9	6.0	10.3
	0.9	0.1	0.5	8.0	5.0	0.9	6.0	10.4
	0.7	0.1	0.8	8.5	4.9	0.9	6.1	10.1
	0.0	0.1	0.0	0.8	0.4	0.0	0.5	1.8
	0.0	0.2**	0.0	0.8	0.3	0.0	0.5	1.7
	0.0	0.2**	0.0	0.8	0.4	0.0	0.5	1.8
	0.0	0.2**	0.0	0.8	0.4	0.0	0.4	2.5

Cystine	Histi-dine	Arginine	Lysine	Hydroxy-lysine*	Aspartic Acid	Glutamic Acid	Total
0.1	0.7	7.4	4.6	0.9	5.5	10.1	96.5
--	0.5	5.7	4.6	0.9	6.0	10.6	93.3
--	0.5	7.8	4.6	0.9	6.1	10.0	98.2
--	0.6	7.7	4.8	0.9	6.5	9.5	101.5
0.1	0.6	8.1	4.3	0.9	5.8	10.4	94.2
0.1	0.6	8.1	4.6	0.9	5.8	10.4	96.3
0.1	0.6	8.0	4.6	0.9	5.8	10.1	95.2
0.1	0.6	8.0	4.8	0.9	5.8	10.7	98.2
0.1	0.7	7.8	4.6	0.9	6.0	10.3	99.1
0.1	0.5	8.0	5.0	0.9	6.0	10.4	102.0
0.1	0.8	8.5	4.9	0.9	6.1	10.1	104.7
0.1	0.0	0.8	0.4	0.0	0.5	1.8	89.4
0.2**	0.0	0.8	0.3	0.0	0.5	1.7**	92.3
0.2**	0.0	0.8	0.4	0.0	0.5	1.8	91.1
0.2**	0.0	0.8	0.4	0.0	0.4	2.5	88.8

GLYCINE

Method: Microbiological assay (65).

Organism: Leuconostoc mesenteroides P-60

Standard: Glycine, A. P. (Amino Acid Manufacturers).

Comment: The range of values for the collagens and gelatins was 23.3-30.5%. The range of values for elastins was 27.9-30.2%. Growth was measured turbidimetrically. The assay values were similar regardless of whether readings were made after 24 hours to 36 hours at 37° C. or 72 hours at 34° C. The values obtained under the same conditions were rather variable. Actually, per cent variation was not great, but the absolute quantity of amino acid involved was of importance. In a recent account of glycine determination with Leuconostoc mesenteroides (66) irregularities were reported. Improvement of the assay values reported in this thesis is planned.

Previous methods: Ester distillation; gravimetric; colorimetric; solubility product.

Reported range of values:

	Chemical	Microbiological (g/100 g.protein)	"Best" Values
Gelatin	16.5-27.0	--	26.0-27.0 (72)
Collagen	6.65-27.2	--	26.2-27.2 (72,73)
Elastin	29.4	--	29.4 (28)

TABLE XVII

GLYCINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Glycine
	%
GELATINS	
Difco	25.7
Calf Skin (Eastman)	26.9
Pig Skin (Eastman)	30.5
Fish Scale	27.4
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	27.5
Cattle Hide (Mild preparation)	29.3
Cattle Bone	23.3
Cattle Achilles Tendon	24.8
Pig Achilles Tendon	26.6
Sheep Achilles Tendon	25.1
Monkey Achilles Tendon	24.4
Chicken Tarso-metatarsal Tendon	26.2
Cattle Tail Tendon	28.0
Rat Tail Tendon	28.9
Kangaroo Tail Tendon	26.4
Turtle Subcutaneous Membrane	26.9
Fish Skin	29.6
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	--
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	29.9
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	--
Cattle Aorta (40 hrs. in 40% urea in 100° C.)	27.9
Sheep Aorta " " " "	30.2
Pig Aorta " " " "	28.8

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

ALANINE

Method: Microbiological assay (71).

Organism: Leuconostoc citrovorum 8081.

Standard: Recrystallized dl-alanine (Merck and Company).

Comment: The medium containing pyridoxine, pyridoxal, and pyridoxamine described by Sauberlich and Bauman (69) was used for the assay. Dense maximum growth was obtained in 18 hours. Growth was measured turbidimetrically. The range of values for gelatins and collagens was 8.6-10.0%. The range of values for all urea-prepared elastins regardless of source was 18.9-20.1%. Elastin purified by 100° C. water instead of urea contained 18.4% alanine.

Previous methods: Ester distillation; gravimetric; oxidation to acetaldehyde; partition chromatography.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100g.protein)	
Gelatin	0.8-9.3	9.0-9.6	8.7-9.6 (74,75,76,69)
Collagen	9.5	---	9.5 (75)
Elastin	0-6.0 (28,77)	---	---

TABLE XVIII

ALANINE CONTENT OF GELATINS, COLLAGENS, and ELASTINS

Sample	Alanine
	%
GELATINS	
Difco	8.6
Calf Skin (Eastman)	8.7
Pig Skin (Eastman)	9.2
Fish Scale	--
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	8.8
Cattle Hide (Mild preparation)	10.0
Cattle Bone	--
Cattle Achilles Tendon	8.7
Pig Achilles Tendon	8.7
Sheep Achilles Tendon	9.1
Monkey Achilles Tendon	8.7
Chicken Tarso-metatarsal Tendon	9.3
Cattle Tail Tendon	8.7
Rat Tail Tendon	9.7
Kangaroo Tail Tendon	8.7
Turtle Subcutaneous Membrane	9.1
Fish Skin	9.2
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	18.4
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	18.9
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	--
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	19.3
Sheep Aorta " " " "	20.0
Pig Aorta " " " "	20.1

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

ISOLEUCINE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: dl-isoleucine (Merck and Company)

Comment: Most of the values for collagen and gelatin were 1.7-2.2%. Gelatins were somewhat low ranging from 1.2-1.9%. Isoleucine appears to be one of the amino acids with greatest variation from one sample to the other. The isoleucine content of elastins was 3.8-4.3% except for pig elastin which contained 2.7%. It may be that the low value for pig elastin is not due to impurity. It will be noted that methionine and lysine content of pig elastin are of the same order as in other elastins. Most proteins for which values have been reported have higher isoleucine content than elastin; so a contaminating protein would be likely to increase rather than decrease assay values.

Previous methods: Oxidation to methylethyl ketone; microbiological.

Reported range of values:

	Chemical	Microbiological (g./100 g.protein)	"Best" Values
Gelatin	1.1	1.4-1.7	1.4-1.7 (78,79)
Collagen	--	--	--
Elastin		3.4 (80)	--

TABLE XIX

ISOLEUCINE CONTENT OF GELATINS, COLLAGENS, and ELASTINS

Sample	Isoleucine
	%
GELATINS	
Difco	1.5
Calf Skin (Eastman)	1.9
Pig Skin (Eastman)	1.5
Fish Scale	1.2
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	2.2
Cattle Hide (Mild preparation)	2.1
Cattle Bone	2.1
Cattle Achilles Tendon	2.1
Pig Achilles Tendon	1.7
Sheep Achilles Tendon	1.7
Monkey Achilles Tendon	
Chicken Tarso-metatarsal Tendon	2.0
Cattle Tail Tendon	1.9
Rat Tail Tendon	1.9
Kangaroo Tail Tendon	
Turtle Subcutaneous Membrane	1.8
Fish Skin	1.7
ELASTINS	
Cattle *Ligamentum nuchae* (24 hrs. in water at 100° C.)	4.3
Cattle *Ligamentum nuchae* (40 hrs. in 40% urea at 100° C.)	4.0
Sheep *Ligamentum nuchae* (24 hours in water at 100° C.)	3.8
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	3.6
Sheep Aorta " " " "	3.9
Pig Aorta " " " "	2.7

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

LEUCINE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: l-leucine (Merck and Company).

Comment: The range of values for collagens and gelatins was 2.5-3.7%. The most frequent values were 3.2-3.5%. Fish scale gelatin was low -2.5% whereas fish collagen contained 3.7%. Elastin contained 7.9-8.7% leucine.

Previous methods: Gravimetric; microbiological; solubility product; oxidation to acetone.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g.protein)	
Gelatin	3.5-7.1	3.2-3.6	3.2-3.6 (81,82,83) (79,84,78)
Collagen	3.5 (83)	--	--
Elastin	--	7.3-8.6 (80,85)	--

TABLE XX

LEUCINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Leucine
	%
GELATINS	
Difco	3.1
Calf Skin (Eastman)	3.1
Pig Skin (Eastman)	3.2
Fish Scale	2.5
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	3.3
Cattle Hide (Mild preparation)	3.2
Cattle Bone	3.4
Cattle Achilles Tendon	3.5
Pig Achilles Tendon	3.4
Sheep Achilles Tendon	3.4
Monkey Achilles Tendon	
Chicken Tarso-metatarsal Tendon	3.4
Cattle Tail Tendon	3.6
Rat Tail Tendon	3.2
Kangaroo Tail Tendon	3.0
Turtle Subcutaneous Membrane	3.5
Fish Skin	3.7
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	8.4
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	8.7
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	8.5
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	8.1
Sheep Aorta " " " "	8.7
Pig Aorta " " " "	7.9

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

VALINE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: dl-valine (Merck and Company).

Comment: The majority of values for valine of collagens and gelatins fell in the range of 2.5-2.8%. Fish scale gelatin was low with 2.2% whereas cattle tail tendon contained 3.2%. Elastin from all sources contained quantities of valine within the limits of 15.5-18.4%. Approximately 18% was found in the majority of samples.

Previous methods: Oxidation to acetone; partition chromatography; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	2.1-3.8	2.5-2.7	2.5-2.7 (82,62,78,79)
Collagen	3.4 (75)	--	--
Elastin	13.5 (28)	13.8 (80)	--

TABLE XXI

VALINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Valine
	%
GELATINS	
Difco	2.8
Calf Skin (Eastman)	2.6
Pig Skin (Eastman)	2.7
Fish Scale	2.2
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	2.5
Cattle Hide (Mild preparation)	2.7
Cattle Bone	2.7
Cattle Achilles Tendon	2.8
Pig Achilles Tendon	2.7
Sheep Achilles Tendon	3.0
Monkey Achilles Tendon	
Chicken Tarso-metatarsal Tendon	2.4
Cattle Tail Tendon	3.2
Rat Tail Tendon	2.9
Kangaroo Tail Tendon	2.7
Turtle Subcutaneous Membrane	2.3
Fish Skin	2.5
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	18.4
Cattle Ligamentum nuchae (40 hrs. at 40% urea at 100° C.)	17.4
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	15.5
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	18.4
Sheep Aorta " " " "	17.6
Pig Aorta " " " "	16.5

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

SERINE AND HYDROXYLYSINE

Method: Periodate oxidation to formaldehyde which is distilled and determined colorimetrically (70).

Standard: Standardized formaldehyde.

Comment: All samples were hydrolyzed 6 hours according to the method of Boyd and Logan (70). Hydrolysis of samples of Difco gelatin for 4 and 6 hours gave essentially the same values. Elastin hydrolyzed for 18 hours yielded values about 10% lower than hydrolysis for 6 hours. Rates of release and destruction of serine (and hydroxylysine) were not determined in detail. However, study of the above data and comparison with that of Boyd and Logan indicated that six hour hydrolysis period was appropriate. Excessive amounts of carbohydrate that might yield formaldehyde are destroyed by this hydrolysis (70). Both serine and hydroxylysine yield formaldehyde. Hydroxylysine is reported (86,75,108) to be approximately 1.0% of gelatin. This hydroxylysine content is assumed for all the samples and the serine content is calculated and expressed in g./100 g. protein in the accompanying table. Elastin is reported to have insignificant quantities (0.0) of hydroxylysine (108) so entire formaldehyde was attributed

to serine. The range of values for gelatin and collagen was 2.8-3.2% except for fish collagen and fish scale gelatin which contained 5.9 and 3.5% respectively. These preparations may be impure, but the value for fish collagen is significantly high. It will be noted that the fish collagen is appreciably high in threonine, another hydroxyamino acid. Microbiological assay for serine offers difficulties. A peculiar S-shaped growth curve with no linear portion is usually obtained. Assays with Cl. perfringens and Leuconostoc mesenteroides yielded unreliable results. The more intensively purified elastins were 0.80-0.82% serine regardless of source.

Previous methods: Gravimetric; periodate oxidation; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	0.4-3.2	3.6-3.8	3.2-3.8 (87,75,88)
Collagen	3.2-3.7	--	3.2-3.7 (75,88)
Elastin	--	--	--

TABLE XXII

SERINE* CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Serine
	%
GELATINS	
Difco	3.2
Calf Skin (Eastman)	2.9
Pig Skin (Eastman)	2.9
Fish Scale	3.5
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	2.8
Cattle Hide (Mild preparation)	2.8
Cattle Bone	3.1
Cattle Achilles Tendon	3.2
Pig Achilles Tendon	3.1
Sheep Achilles Tendon	3.0
Monkey Achilles Tendon	
Chicken Tarso-metatarsal Tendon	2.8
Cattle Tail Tendon	3.2
Rat Tail Tendon	3.2
Kangaroo Tail Tendon	3.1
Turtle Subcutaneous Membrane	
Fish Skin	5.9
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	1.00
Cattle Ligamentum nuchae (40 hrs. at 40% urea at 100° C.)	0.82
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	0.80
Sheep Aorta " " " "	
Pig Aorta " " " "	0.82

The Term "per cent" means g. amino acid per 100 g. moisture-free protein.

* All gelatins and collagens were assumed to contain 1.0% hydroxylysine. Serine values were calculated on this basis.

THREONINE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: dl-threonine (Merck and Company)

Comment: The best values for threonine of the collagens was 2.2-2.3%. Turtle and fish collagen and fish scale gelatin formed a special group containing 3.3-3.5% threonine. It will be recalled that fish collagen was unusually high in serine content (plus hydroxylysine). These amino acids are all hydroxyamino acids. The predominant values for threonine content of elastins was 1.0-1.2%.

Previous methods: Periodate oxidation; lead tetra-acetate oxidation; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	0.5-2.2	1.9-2.0	1.9-2.2 (78,79,75,88)
Collagen	2.3-2.4		2.3-2.4 (75,88)
Elastin	--	1.1	1.1 (80)

TABLE XXIII

THREONINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Threonine
	%
GELATINS	
Difco	2.0
Calf Skin (Eastman)	2.2
Pig Skin (Eastman)	2.2
Fish Scale	3.3
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	2.2
Cattle Hide (Mild preparation)	2.2
Cattle Bone	2.3
Cattle Achilles Tendon	2.3
Pig Achilles Tendon	2.3
Sheep Achilles Tendon	2.5
Monkey Achilles Tendon	2.3
Chicken Tarso-metatarsal Tendon	2.5
Cattle Tail Tendon	2.3
Rat Tail Tendon	2.5
Kangaroo Tail Tendon	2.3
Turtle Subcutaneous Membrane	3.4
Fish Skin	3.5
ELASTINS	
Cattle *Ligamentum nuchae* (24 hours in water at 100° C.)	1.15
Cattle *Ligamentum nuchae* (40 hrs. in 40% urea at 100° C.)	0.96
Sheep *Ligamentum nuchae* (24 hrs. in water at 100° C.)	1.6
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	1.16
Sheep Aorta " " " "	1.01
Pig Aorta " " " "	1.31

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

PROLINE

Method: Microbiological assay (68,65,66)

Organism: Leuconostoc mesenteroides P-60

Standard: l-proline (Pfanstiehl)

Comment: Growth was measured turbidimetrically after 24-36 hours at 37° C. on the medium of Henderson and Snell (65), 72 hours at 34° C. on the medium of Dunn et al. (68), and 20 hours at 37° C. on the medium of Steele et al. (66). The best procedure was apparently that of Steele et al. Growth was faster and greater on this medium. The range of values for collagen and gelatin was 13.3-16.3% proline. Elastin from all sources contained 15.4-17.0% proline.

Previous methods: Gravimetric; partition chromatography; solubility product; oxidation to pyrrole.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	6.3-26.5	---	14.8-17.6 (72,89,90,75)
Collagen	15.1-26.5	---	15.1-17.5 (75,90,72)
Elastin	15.2 (28)	15.6 (80)	15.2-15.6

TABLE XXIV

PROLINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Proline
	%
GELATINS	
Difco	16.3
Calf Skin (Eastman)	14.0
Pig Skin (Eastman)	16.3
Fish Scale	14.1
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	15.8
Cattle Hide (Mild preparation	16.5
Cattle Bone	--
Cattle Achilles Tendon	13.3
Pig Achilles Tendon	14.3
Sheep Achilles Tendon	14.1
Monkey Achilles Tendon	--
Chicken Tarso-metatarsal Tendon	15.7
Cattle Tail Tendon	14.9
Rat Tail Tendon	15.5
Kangaroo Tail Tendon	--
Turtle Subcutaneous Membrane	15.6
Fish Skin	13.7
ELASTINS	
Cattle Ligamentum nuchae (24 hours in water at 100° C.)	--
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	17.0
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	--
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	15.8
Sheep Aorta " " " "	16.8
Pig Aorta " " " "	15.4

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

HYDROXYPROLINE

Method: Oxidation with H_2O_2 to pyrrole which is determined colorimetrically (71).

Standard: l-hydroxyproline (Pfanstiehl)

Comment: The hydrolysates used in this determination were those prepared for the microbiological assays. Results from this method varied widely from one determination to another. In general, values obtained were low compared with those reported. The assay values showed a characteristic decline at higher levels of the standard curve, although the standard curves were linear. Hydrolysates of elastin also showed lower than the reported content of hydroxyproline as well as the characteristic decline at higher levels. The synthetic mixture of component amino acids and NaCl comparing with gelatin hydrolysates yielded results identical with the pure hydroxyproline standard. Hydrolysates of Difco gelatin were also prepared by autoclaving 0.5 g. gelatin with 10.0 ml. 3 M HCl at 45 pounds for 12 and 30 hours. These yielded the same effects and values as the microbiological assay hydrolysates.

Further study of this method is planned. The values presented are recognized as unreliable.

Qualitatively they show the presence of hydroxyproline and afford some comparison of samples.

Previous methods: Gravimetric; colorimetric.

Reported range of Values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	6.4-14.7	---	14.1-14.7 (74,91,71,92)
Collagen	10.8 (73)	---	---
Elastin	2.0 (28)	---	---

TABLE XXV

HYDROXYPROLINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Hydroxyproline
	%
GELATINS	
Difco	8.5
Calf Skin (Eastman)	--
Pig Skin (Eastman)	9.7
Fish Scale	--
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	13.5
Cattle Hide (Mild preparation)	8.5
Cattle Bone	--
Cattle Achilles Tendon	10.3
Pig Achilles Tendon	6.4
Sheep Achilles Tendon	--
Monkey Achilles Tendon	--
Chicken Tarso-metatarsal Tendon	10.6
Cattle Tail Tendon	--
Rat Tail Tendon	8.4
Kangaroo Tail Tendon	10.0
Turtle Subcutaneous Membrane	--
Fish Skin	--
ELASTINS	
Cattle Ligamentum nuchae (24 hours in water at 100° C.)	--
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	--
Sheep Ligamentum nuchae (24 hours in water at 100° C.)	--
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	--
Sheep Aorta " " " "	--
Pig Aorta " " " "	0.9

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

PHENYLALANINE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: dl-phenylalanine (Merck and Company).

Comment: Most values for phenylalanine of the wide variety of collagens were 2.3-2.5%. Two of the commercial gelatins were a little low according to this standard. It is noteworthy that tyrosine values for these two gelatins were low also. The elastins subjected to most intensive purification were in the majority of cases 5.0-5.1% phenylalanine regardless of source.

Previous methods: Ester distillation; colorimetric; gravimetric; partition chromatography; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	0.4-2.6	2.2-2.3	2.2-2.6 (93,94,95,78, 79,75)
Collagen	4.2 (75)	---	---
Elastin	2.23 (96)	4.8 (80)	---

TABLE XXVI

PHENYLALANINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Phenylalanine
	%
GELATINS	
Difco	2.3
Calf Skin (Eastman)	1.9
Pig Skin (Eastman)	2.1
Fish Scale	2.2
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	2.3
Cattle Hide (Mild preparation)	2.4
Cattle Bone	2.6
Cattle Achilles Tendon	2.4
Pig Achilles Tendon	2.4
Sheep Achilles Tendon	2.3
Monkey Achilles Tendon	2.4
Chicken Tarso-metatarsal Tendon	2.6
Cattle Tail Tendon	2.4
Rat Tail Tendon	2.6
Kangaroo Tail Tendon	2.5
Turtle Subcutaneous Membrane	
Fish Skin	2.4
ELASTINS	
Cattle Ligamentum nuchae (24 hours in water at 100° C.)	5.7
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	5.0
Sheep Ligamentum nuchae (24 hours in water at 100° C.)	5.4
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	5.1
Sheep Aorta " " " "	5.0
Pig Aorta " " " "	5.4

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

TYROSINE

Method: Microbiological assay (63)

Organism: Cl. perfringens BP6K.

Standard: l-tyrosine (Merck and Company).

Comment: Most values for tyrosine of the wide variety of collagens were 0.9-1.0%. One commercial gelatin, Eastman calf skin gelatin is significantly low in tyrosine. The different values from gelatin to gelatin illustrate the unreliability of collagen values based on commercial gelatin preparations. Best tyrosine values for elastin are 1.6-2.0%. An appreciably different quantity is found in pig aorta elastin preparation, which contains 2.9%. This may represent impurity.

Previous methods: Gravimetric; colorimetric; partition chromatography; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	0-1.1	0.44	0.49-1.1 (78,76)
Collagen	0.8-1.4	--	1.0 (97,18)
Elastin	1.6 (28)	1.4 (80)	---

TABLE XXVII

TYROSINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Tyrosine
	%
GELATINS	
Difco	0.91
Calf Skin (Eastman)	0.14
Pig Skin (Eastman)	0.69
Fish Scale	0.72
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	0.89
Cattle Hide (Mild preparation)	1.00
Cattle Bone	0.98
Cattle Achilles Tendon	0.91
Pig Achilles Tendon	0.96
Sheep Achilles Tendon	0.89
Monkey Achilles Tendon	1.02
Chicken Tarso-metatarsal Tendon	0.90
Cattle Tail Tendon	0.98
Rat Tail Tendon	1.06
Kangaroo Tail Tendon	0.93
Turtle Subcutaneous Membrane	0.97
Fish Skin	0.86
ELASTINS	
Cattle Ligamentum nuchae (24 hours in water at 100° C.)	1.85
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	1.61
Sheep Ligamentum nuchae (24 hours in water at 100° C.)	2.36
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	1.63
Sheep Aorta " " " "	2.10
Pig Aorta " " " "	2.91

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

TRYPTOPHAN

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: l-tryptophan (Merck and Company).

Comment: Hydrolysis of sample was with 5.0 N NaOH according to the method of Stokes et al. (79). All samples contained minor traces of tryptophan. Tryptophan added to hydrolysates was completely recovered, thus showing absence of inhibition. Tryptophan appears to be a contaminant of collagen and elastin.

Previous methods: Colorimetric; absorption spectrophotometry; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	0.0-0.12	0.003-0.021	0.0 (98,81)
Collagen	0.0	---	0.0 (14)
Elastin	0.0	---	0.0 (28)

TABLE XXVIII

TRYPTOPHAN CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Tryptophan
	%
GELATINS	
Difco	
Calf Skin (Eastman)	
Pig Skin (Eastman)	
Fish Scale	
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	
Cattle Hide (Mild preparation)	
Cattle Bone	<0.01
Cattle Achilles Tendon	<0.01
Pig Achilles Tendon	<0.01
Sheep Achilles Tendon	<0.01
Monkey Achilles Tendon	
Chicken Tarso-metatarsal Tendon	<0.01
Cattle Tail Tendon	
Rat Tail Tendon	
Kangaroo Tail Tendon	
Turtle Subcutaneous Membrane	
Fish Skin	
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	<0.01
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	<0.01
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	
Sheep Aorta " " " "	
Pig Aorta " " " "	

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

METHIONINE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: dl-methionine (Merck and Company).

Comment: Most values for methionine of the gelatins and collagens were 0.80-1.0%. Fish collagen and fish scale gelatin, containing 2.2-2.3% were sharply in contrast to other samples. It is interesting that these samples should resemble each other while turtle collagen resembles the larger group of collagens. Pure elastin may be said to lack methionine.

Previous methods: Colorimetric; partition chromatography; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	0.8-1.0	0.59	0.6-1.0 (81,18,100,101)
Collagen	0.8		0.8 (14)
Elastin	0.38 (28)	0.3 (80)	---

TABLE XXIX

METHIONINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Methionine
	%
GELATINS	
Difco	0.92
Calf Skin (Eastman)	0.85
Pig Skin (Eastman)	0.80
Fish Scale	2.2
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	0.89
Cattle Hide (Mild preparation)	0.94
Cattle Bone	0.72
Cattle Achilles Tendon	0.84
Pig Achilles Tendon	0.89
Sheep Achilles Tendon	0.86
Monkey Achilles Tendon	0.98
Chicken Tarso-metatarsal Tendon	1.16
Cattle Tail Tendon	0.89
Rat Tail Tendon	0.96
Kangaroo Tail Tendon	0.91
Turtle Subcutaneous Membrane	0.68
Fish Skin	2.3
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	0.03
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	0.03
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	0.12
Sheep Aorta (40 hrs. in 40% urea at 100° C.)	0.00
Cattle Aorta " " " "	0.00
Pig Aorta " " " "	0.00

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

CYSTINE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: l-cystine (Merck and Company).

Comment: All the collagens and gelatins contained traces of cystine. The most common values were 0.08-0.12%. Fish scale gelatin contained 0.17% cystine. The two samples of hide collagen may have given erroneously low values for cystine. These samples were ground in a Wiley mill which forces the particles through a brass screen. To determine cystine, 40-50 mg. of hydrolyzed protein was necessary to obtain sufficient growth. Possibly inhibitory amounts of copper for the amount of growth obtained was contained in 40-50 mg. of protein. A sample of rat tail tendon washed with water from a copper tank revealed this effect. A companion sample of rat tail tendon washed with distilled water directly from the still yielded 0.08% cystine. The greater growth obtained with smaller amounts of protein hydrolysate in assays for other amino acids seemed to obviate the copper inhibition. Elastin contained 0.15-0.25% cystine.

Previous methods: Nitrogen distribution; colorimetric; manometric.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	0.00-0.31	---	0.1-0.2 (81,99)
Collagen	---	---	---
Elastin	0.23 (28)	0.6 (80)	---

TABLE XXX

CYSTINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Cystine
	%
GELATINS	
Difco	0.09
Calf Skin (Eastman)	0.05
Pig Skin (Eastman)	0.09
Fish Scale	0.17
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	0.04
Cattle Hide (Mild preparation	0.02
Cattle Bone	0.11
Cattle Achilles Tendon	0.11
Pig Achilles Tendon	0.10
Sheep Achilles Tendon	0.10
Monkey Achilles Tendon	
Chicken Tarso-metatarsal Tendon	0.12
Cattle Tail Tendon	
Rat Tail Tendon	0.08
Kangaroo Tail Tendon	0.10
Turtle Subcutaneous Membrane	
Fish Skin	0.12
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	0.25
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	0.15
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	
Sheep Aorta " " " "	
Pig Aorta " " " "	

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

HISTIDINE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: l-histidine·H_2O·HCl (Merck and Company)

Comment: The range of histidine content for gelatins and collagens was 0.56-1.01%. Amino acids present in small amounts in a sample may show wide per cent variations from sample to sample; impurities present may change the amount of the amino acid out of proportion to that present in the pure protein. Elastin from cattle ligamentum nuchae can be considered to lack histidine.

Previous methods: Nitrogen distribution; colorimetric; gravimetric; microbiological; electrodialysis.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	0.53-1.2	0.58-0.79	0.6-1.0 (74,94,81,78,79, 75,102)
Collagen	0.30-0.9		0.6-0.9 (73,18,75,102)
Elastin	0.0 (28)	0.04 (80)	0.0

TABLE XXXI

HISTIDINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Histidine
	%
GELATINS	
Difco	0.85
Calf Skin (Eastman)	0.63
Pig Skin (Eastman)	0.67
Fish Scale	1.01
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	0.62
Cattle Hide (Mild preparation)	0.74
Cattle Bone	0.64
Cattle Achilles Tendon	0.75
Pig Achilles Tendon	0.66
Sheep Achilles Tendon	0.67
Monkey Achilles Tendon	
Chicken Tarso-metatarsal Tendon	0.68
Cattle Tail Tendon	0.80
Rat Tail Tendon	0.56
Kangaroo Tail Tendon	
Turtle Subcutaneous Membrane	0.68
Fish Skin	0.94
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	0.05
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	0.07
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	0.21
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	
Sheep Aorta " " " "	
Pig Aorta " " " "	

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

ARGININE

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: l-arginine·HCl (Merck and Company).

Comment: Most values for gelatins and collagens were 8.6-9.0%. It has been shown that alkaline treatment of collagen partially destroys arginine (61). It will be noted that Eastman calf skin gelatin was significantly low in arginine; Difco gelatin was somewhat low. Further evidence is thus provided that commercial samples of gelatin may vary widely in composition and hence fail to represent its collagen precursor. Elastins contained 0.87-1.05% arginine.

Previous methods: Nitrogen distribution; gravimetric; arginase; colorimetric; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	7.4-8.7	8.0-9.3	8.6-9.3 (102,75,62,103)
Collagen	7.1-8.8	---	8.8 (75,102)
Elastin	1.0 (28)	1.1 (80)	---

TABLE XXXII

ARGININE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Arginine
	%
GELATINS	
Difco	8.3
Calf Skin (Eastman)	6.4
Pig Skin (Eastman)	8.8
Fish Scale	—
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	8.7
Cattle Hide (Mild preparation)	8.6
Cattle Bone	9.0
Cattle Achilles Tendon	9.0
Pig Achilles Tendon	9.0
Sheep Achilles Tendon	8.9
Monkey Achilles Tendon	8.6
Chicken Tarso-metatarsal Tendon	8.9
Cattle Tail Tendon	8.7
Rat Tail Tendon	8.9
Kangaroo Tail Tendon	8.6
Turtle Subcutaneous Membrane	9.0
Fish Skin	9.5
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	1.05
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	0.89
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	0.87
Sheep Aorta " " " "	0.90
Pig Aorta " " " "	0.93

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

LYSINE

Method: Microbiological assay (68,65,66).

Organism: Leuconostoc mesenteroides P-60.

Standard: l-lysine·H_2O·HCl (Merck and Company).

Comment: Assays conducted with the medium of Dunn et al. (68) gave approximately the same values as did those on the medium of Henderson and Snell (65) or on the medium of Steele et al. (66). Growth was twice as fast and in amount on the medium of Steele et al. as on the Henderson and Snell medium and five times that on the medium of Dunn et al. as measured turbidimetrically. All growth was measured turbidimetrically after incubation for 72 hours at 34° C. on Dunn's medium, 24-36 hours at 37° C. on Henderson and Snell's medium, and 20 hours at 37° C. on the medium of Steele et al. Collagens and gelatins contained lysine in the range of 4.9-5.7%. Elastins from all sources contained 0.37-0.51% lysine

Previous methods: Nitrogen distribution; gravimetric; microbiological; electrodialysis; amino nitrogen determination.

Reported Range of values

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	4.1-8.0	4.1-6.9	4.1-5.9 (81,78,75,74,94, 102,104)
Collagen	3.8-9.4	---	4.1-5.0 (105,73,18,75, 102)
Elastin	0 (28)	0.5 (80)	---

TABLE XXXIII

LYSINE CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Lysine
	%
GELATINS	
Difco	5.2
Calf Skin (Eastman)	5.2
Pig Skin (Eastman)	5.1
Fish Scale	4.9
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	5.3
Cattle Hide (Mild preparation)	5.5
Cattle Bone	5.5
Cattle Achilles Tendon	4.9
Pig Achilles Tendon	5.2
Sheep Achilles Tendon	5.3
Monkey Achilles Tendon	5.1
Chicken Tarso-metatarsal Tendon	5.5
Cattle Tail Tendon	5.3
Rat Tail Tendon	5.7
Kangaroo Tail Tendon	5.5
Turtle Subcutaneous Membrane	5.1
Fish Skin	
ELASTINS	
Cattle *Ligamentum nuchae* (24 hrs. in water at 100° C.)	
Cattle *Ligamentum nuchae* (40 hrs. in 40% urea at 100° C.)	0.39
Sheep *Ligamentum Nuchae* (24 hrs. in water at 100° C.)	0.42
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	0.47
Sheep Aorta " " " "	0.37
Pig Aorta " " " "	0.51

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

ASPARTIC ACID

Method: Microbiological assay (67,65,66).

Organism: Leuconostoc mesenteroides P-60.

Standard: l-aspartic acid, A. P. (Amino Acid Manufacturers, University of California).

Comment: Growth on the medium of Steele et al. (66) was equivalent to that on yeast-peptone medium. Assay values obtained with turbidimetric readings after 20 hours at 37° C. on this medium were the same as those obtained turbidimetrically after 72 hours growth at 34° C. on the medium of Hac and Snell (67), or 24-36 hours at 37° C. on the medium of Henderson and Snell (65). The range of values for gelatin and collagen was 6.3-7.5% aspartic acid. The range of values for elastin was 0.61-0.63% except for pig elastin which contained 0.44%.

Previous methods: Gravimetric; ester distillation; microbiological.

Reported range of values:

	Chemical	Microbiological (g./100 g. protein)	"Best" Values
Gelatin	0.6-3.4	5.5-6.8	5.5-6.8 (67,87)
Collagen	3.4-6.3	---	6.3 (14)
Elastin	0.0 (28)	0.6 (80)	0.6

TABLE XXXIV

ASPARTIC ACID CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Aspartic Acid %
GELATINS	
Difco	6.4
Calf Skin (Eastman)	6.9
Pig Skin (Eastman)	6.3
Fish Scale	6.5
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	7.0
Cattle Hide (Mild preparation)	7.5
Cattle Bone	—
Cattle Achilles Tendon	6.7
Pig Achilles Tendon	6.7
Sheep Achilles Tendon	6.7
Monkey Achilles Tendon	7.0
Chicken Tarso-metatarsal Tendon	6.7
Cattle Tail Tendon	6.9
Rat Tail Tendon	6.9
Kangaroo Tail Tendon	7.0
Turtle Subcutaneous Membrane	5.2
Fish Skin	6.5
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	—
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	0.63
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	—
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	0.61
Sheep Aorta " " " "	0.64
Pig Aorta " " " "	0.44

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

GLUTAMIC ACID

Method: Microbiological assay (63).

Organism: Cl. perfringens BP6K.

Standard: l-glutamic acid (Merck and Company).

Comment: The gelatins and collagens yielded values of 10.8-12.2%. glutamic acid. Elastins yielded 1.9-2.2% glutamic acid except in the case of pig elastin which contained 2.8% glutamic acid, perhaps resulting from impurity. Glutamic acid standard curves were S-shaped with a distinct lag. Addition of small quantities of glutamine (50-200 μg./tube) or of NH_4Cl (10 mg./tube) did not appreciably alter the curve, nor did withdrawal of the aspartic acid of the medium have significant effect.

Previous methods: Ester distillation; gravimetric; conversion to pyrrollidone carboxylic acid; microbiological.

Reported range of values:

	Chemical	Microbiological	"Best" Values
		(g./100 g. protein)	
Gelatin	1.8-11.7	10.2-11.5	10.2-11.7 (106,107,67,78)
Collagen	5.7-11.3	---	10.2-11.3 (14,93)
Elastin	---	3.3 (80)	---

TABLE XXXV

GLUTAMIC ACID CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Glutamic Acid %
GELATINS	
Difco	11.5
Calf Skin (Eastman)	12.1
Pig Skin (Eastman)	11.7
Fish Scale	11.4
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	11.4
Cattle Hide (Mild preparation)	10.8
Cattle Bone	11.7
Cattle Achilles Tendon	11.9
Pig Achilles Tendon	11.9
Sheep Achilles Tendon	11.5
Monkey Achilles Tendon	--
Chicken Tarso-metatarsal Tendon	12.2
Cattle Tail Tendon	11.7
Rat Tail Tendon	11.9
Kangaroo Tail Tendon	11.6
Turtle Subcutaneous Membrane	11.9
Fish Skin	11.5
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	2.2
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	2.1
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	1.9
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	2.1
Sheep Aorta " " " "	--
Pig Aorta " " " "	2.8

The term "per cent" means g. amino acid per 100 g. moisture-free protein.

NITROGEN CONTENT OF GELATIN, COLLAGEN, AND ELASTIN

Previously reported nitrogen content has been:

Gelatin - 15.4-18.4% (100,94)

Collagen - 17.2-18.6% (14)

Elastin - 16.6-17.1% (28,80)

The variations might be due to difference in preparatory procedures or to accuracy of the nitrogen determination.

To obtain the data in the accompanying table, three digestion catalysts were compared - $CuSO_4$, HgO, and $CuSO_4$ + $SeOCl_2$. All gave the same values on the test samples when digestion was continued 6-10 hours after clearing. Digestion for 3 hours after clearing gave values 2-5% lower than digestion for 6 hours. Digestion for 10 hours gave the same values as digestion for 6 hours.

The digestion procedure finally chosen was that used by Bowes and Kenton (14) to determine the nitrogen content of their "native" collagen. The digestion mixture consisted of 1.6 g. Na_2SO_4, 4.0 g. $CuSO_4 \cdot 5H_2O$, and 8.0 mg. Se (in the form of $SeOCl_2$ instead of Na_2SeO_3)/10.0 ml. H_2SO_4. Digestion time was 6 hours after clearing. The recovery of 50 mg. lysine HCl with this procedure was 95%. The nitrogen content of "native" collagen prepared similarly to the method of Bowes and Kenton yielded 18.1% nitrogen. Two of three samples of elastin yielded 17.1% nitrogen which agrees with

the highest value reported for this protein.

The accompanying table shows that the range of values for gelatins was 17.4–18.0%; collagens, 17.8–18.4%; and elastins, 16.7–17.1%.

TABLE XXXVI

NITROGEN CONTENT OF GELATINS, COLLAGENS, AND ELASTINS

Sample	Nitrogen
	%
GELATINS	
Difco	17.6
Calf Skin (Eastman)	17.4
Pig Skin (Eastman)	18.0
Fish Scale	17.6
COLLAGENS	
Cattle Hide (Prepd. with $Ca(OH)_2$ + trypsin)	17.8
Cattle Hide (Mild preparation)	18.1
Cattle Bone	18.0
Cattle Achilles Tendon	18.1
Pig Achilles Tendon	18.1
Sheep Achilles Tendon	17.9
Monkey Achilles Tendon	
Chicken Tarso-metatarsal Tendon	17.8
Cattle Tail Tendon	17.8
Rat Tail Tendon	18.2
Kangaroo Tail Tendon	17.8
Turtle Subcutaneous Membrane	
Fish Skin	18.4
ELASTINS	
Cattle Ligamentum nuchae (24 hrs. in water at 100° C.)	16.7
Cattle Ligamentum nuchae (40 hrs. in 40% urea at 100° C.)	17.1
Sheep Ligamentum nuchae (24 hrs. in water at 100° C.)	--
Cattle Aorta (40 hrs. in 40% urea at 100° C.)	17.1
Sheep Aorta " " " "	--
Pig Aorta " " " "	--

SUMMARY

Collagen and elastin were prepared from a number of different species of animals and tissues. Rates of solubilization and denaturation and extent of swelling varied widely for the different collagens. Trypsin, chymotrypsin, and papain inappreciably digested the collagens. Pepsin, Cl. perfringens enzymes, and Cl. histolyticum filtrates extensively solubilized the collagens regardless of source.

Heat denaturation of cattle achilles tendon collagen increased susceptibility to attack by trypsin, papain, and Cl. perfringens to the same extent. Urea denaturation had a similar effect. Care must be taken that substrates used in detection of "collagenases" are not modified in preparation.

Collagens from several sources demonstrated greatly enhanced ease of solubilization after trypsin or papain pretreatment. This effect was shown to be due to residual enzyme.

Elastin, in contrast to collagen, was solubilized by trypsin and papain. Chymotrypsin, pepsin, Cl. perfringens enzymes and Cl. histolyticum enzymes apparently had little action on elastin. Heating at 100° C. in water or 40% urea solutions somewhat increased the susceptibility of elastin to trypsin, papain, and pepsin.

An almost complete amino acid assay of four gelatin, thirteen collagen and six elastin preparations was made.

In general, the amino acid composition was uniform for gelatins and collagens regardless of source. One commercial gelatin was relatively low in arginine and tyrosine. Fish skin collagen contained 50-100% more methionine, serine, and threonine than most of the other collagens. Turtle subcutaneous membrane collagen resembled fish skin collagen in threonine content. Fish scale gelatin was like fish skin collagen in threonine and methionine content.

Elastins exhibited uniformity of amino acid composition from one preparation to another except in the case of pig aorta elastin which was relatively low in isoleucine and high in tyrosine and glutamic acid.

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