substrate during the reaction; wavelength, 284 m μ (or within the range 284–290 m μ); initial substrate concentration, within the range 0.1–0.3 mg./ml.; slit width as narrow as possible. The final decision on slit width will depend on the conditions of the experiment and the sensitivity and stability of the spectrophotometer. The most important factor is that it should remain constant throughout a set of measurements.

SUMMARY

1. A spectrophotometric assay of bovine ribonuclease has been developed based on the change in ultraviolet-absorption spectrum resulting from the hydrolysis of cytidine 2':3'-phosphate.

2. The relevant optical and kinetic details have been carefully investigated and satisfactory conditions for the assay have been delineated.

3. Simple modifications which allow rapid assays of somewhat lower precision are also described.

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Structural Changes in Collagen

THE ACTION OF ALKALIS AND ACIDS IN THE CONVERSION OF COLLAGEN INTO EUCOLLAGEN

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The chemical and structural changes which the collagen of native adult mammalian skin, bone and tendon can undergo are difficult to follow because of the insolubility of the protein and the presence in small quantities of other substances which can be completely eliminated only by reagents which may modify the collagen. Reaction with 1-fluoro-2:4-dinitrobenzene is one of the most satisfactory methods of following the changes, since it can indicate the release of free α -amino groups. These may be formed either from the rupture of peptide bonds, from the removal of substances masking the groups or from changes of configuration which remove steric difficulties (see Steven & Tristram, 1958, for lysine ϵ -amino groups). Bowes & Moss (1953) and Grassmann & Hörmann (1953) have shown native ox-hide collagen to be without detectable *N*-terminal residues, whereas small quantities of end groups have been shown to be released by certain treatments. Bowes & Moss (1953), after using 8M-urea, found a small amount of *N*-terminal aspartic acid (1 mole/1400 kg. of collagen) and, after using alkali at pH 13.0 for 14 days, found *N*-terminal aspartic acid, glutamic acid, phenylalanine and glycine, with a residue total of 1 mole/130 kg. of collagen. Deasy (1958),

with a similar collagenous material, found *N*-terminal aspartic acid, serine and glycine in small quantities after a very mild treatment at about pH 8.6.

The conversion of collagen into gelatin of high molecular weight often requires, as an initial stage, the prior treatment of collagen in alkali or acid. The quantities of *N*-terminal residues found in gelatins by Courts (1954*a*) are substantially in excess of those given above for collagen, and Courts (1958) showed, in an extreme case, that the severe changes in protein chain length, which take place in the overall process whereby collagen is converted into gelatin, occur mainly during the initial pretreatment with cold alkali rather than as a result of hydrolyses during hot extraction of the gelatin. The present work describes in more detail the changes brought about in collagen by alkalis and acids with *N*-terminal-residue assay as the principal means of assessing changes.

Parallel with the changes in collagen during pretreatment is the solution in the alkaline and acid reagents used of substances which may play a part in the stability of the structure of native collagen. Partridge (1948) suggested that chondroitin sulphate in cartilaginous tissue acted as a multivalent

ion, building up complexes with protein molecules in the form of fibrous macromolecules. Jackson (1953) has pointed out that conditions which favour mucoprotein extraction also lead to a striking reduction in tissue stability. In this paper he refers solely to alkaline reagents and calcium chloride, but in a later paper (1954) he shows similar results with hyaluronidase. Acid conditions, which are considered to be unfavourable to mucoprotein extraction, but which may impair collagen stability, have previously only been studied empirically (Ames, 1957; Kuntzel, Cars & Heidemann, 1958) but are included with alkalis in this report.

Ames (1957) suggested the possibility of obtaining a purified collagen, and hence a purified gelatin, by successive extractions of collagen with acid and alkali in the cold, thus benefiting by the removal of those non-collagen impurities which are soluble in one but not the other pretreatment. His results with ox-tendon, which were essentially exploratory, showed a secondary effect; the initial hydrochloric acid treatment apparently opened up the structure for subsequent calcium hydroxide attack. The reaction of bone collagen to this type of 'dual-soak process' has been assessed, together with the other reactions, in this present study and found to have important implications.

The evidence for multi-chain molecules (Courts & Stainsby, 1958) suggests that the number-average molecular weight (M_n) values calculated from *N*-terminal residue assay (Courts, 1954*a, b*) should be more correctly termed 'number-average chain weight' (C_n). Thus if 100 000 g. of protein gives a total of *T* mole of *N*-terminal residues, then C_n is 100 000/*T*.

It is clear from Courts (1958), Courts & Stainsby (in preparation) and the present work that collagen which has been subjected to intensive alkali treatment in the presence of a restrainer of swelling is considerably different in many of its properties from native collagen, so that the retention of the term collagen for pretreated material may be misleading. The number-average chain weight (C_n) of well pretreated collagen is about 62 000, which is similar to gelatin rather than to native collagen. In this paper the term 'eucollagen' (Gr.: easily producing glue) has been adopted. 'Eucollagen' is defined as collagen which has been chemically modified so as to be transformed completely into gelatin, with C_n 60 000 (approx.), in a few minutes below 60° in water at pH 7.0, or in hydrogen-bond breakers at lower temperatures, e.g. at 18° with 2*M*-sodium salicylate, 2*M*-potassium thiocyanate, 4*M*-lithium bromide and 4*M*-mercaptoacetic acid, as described by Courts (1958). This implies that only secondary forces, including hydrogen bonds, are retaining the collagen configuration. A further property of 'eucollagen', which emphasizes its

structural remoteness from native adult collagen, is its ready solubility in dilute acid buffers, giving rise to a solution which is conveniently termed 'soluble eucollagen'.

MATERIALS AND METHODS

Bone collagen (ossein). The most convenient form of adult mammalian collagen for the present work, available in quantity, was ossein. This was obtained as a commercial product prepared from the mixed bones of the Indian ox. The bones had been sun-dried and demineralized with HCl for about 1 week at 10–15°, a countercurrent process being used; spent acid is used initially on the bone, then the acid concentration is raised to a final value of 8% (w/v). In the present work ossein from the compact bone was used and the spongy pieces were rejected. Ossein pieces are irregular in shape, and, in the batch used, they had an average particle volume of approx. 0.15 ml., as estimated by the displacement of water method. This material had an ash content of 0.6% and water content of 20%.

Ash content. Ash contents were determined as sulphated ash by heating for 16 hr. at 550° initially in the presence of H_2SO_4 .

Water content. The water (or moisture) content was determined by drying to constant weight at 105°.

N-Terminal residue determinations. (a) Reaction with 1-fluoro-2,4-dinitrobenzene (FDNB). The method of Sanger (1945) was used as modified for gelatin by Courts (1954*a*). The procedure for the collagens was very similar. These materials were washed to an approximately neutral pH value and finely subdivided in a food disintegrator at 1800 rev./min. before the reaction with FDNB. Collagen or eucollagen, containing about 2 g. of protein, was suspended in 4% $NaHCO_3$ (200 ml.), treated with excess of FDNB (5.7 g.) at 25° and vigorously shaken for 24 hr. The 2,4-dinitrophenyl (DNP)-protein was strained off on nylon mesh and washed as follows: 5 min. in water (500 ml.), 2 hr. in 0.1 *N*-HCl (200 ml.), then 2 hr. in water followed by several treatments with acetone until no further yellow colour was discharged. The solid was air-dried at room temperature. Ash and water contents were determined.

(b) Hydrolysis. About 500 mg. of DNP-protein was heated with 5.5 *N*-HCl (5 ml.) for 16 hr. in sealed tubes at 100°. The extraction of DNP-amino acids, their separation and identification by chromatography on Celite and the quantitative estimations were carried out as described in Courts (1954*a*), except that, in view of the conclusions in that paper and in Grassmann & Hörmann (1953) and Heyns & Legler (1956), no attempts were made to find *N*-terminal proline or hydroxyproline.

Thermal conversions into gelatin. These were carried out at approx. pH 7 in water. The treated collagen was heated at 60° for 2 hr., the residue was heated at 80° for 3 hr. and that residue heated at 93° for 2 hr. The gelatin solutions were filtered hot and gelled at 4°. Strips of gel approx. 4 cm. × 1 cm. × 0.5 cm. were dried in a current of air at 20–30°. Moisture and ash contents were measured.

Collagen to eucollagen reactions. The two alkalis chosen for this study were (A) saturated $Ca(OH)_2$ solution, which maintains pH 12.6 at room temperature and may therefore be considered to give mild alkaline treatment to collagen, and (B) NaOH solutions at pH values about 14, representing more intensive alkaline attack. The pH-swelling curves

for collagen (Bowes & Kenten, 1950) suggest that treatment (A) will not lead to excessive swelling and treatment (B) will be objectionable because of the high degree of swelling. Treatment (B) produces fragile collagen, not uniformly treated throughout the mass and difficult to wash salt-free at pH 7.0. In addition the outer fibres dissolve to give low-molecular-weight protein. Bowes & Kenten (1950) showed that the presence of 2M-NaCl restrained the swelling in the range pH 10–13 but not above. Kuntzel *et al.* (1958) found that acid swelling was almost eliminated by either NaCl or Na_2SO_4 . The latter compound was used effectively by Ames (1944) and A. G. Ward (unpublished work) in swelling experiments on collagen at high pH values and was also adopted in the present work. The following experiments were designed to give increasing degrees of pretreatment.

Calcium hydroxide solution. Collagen (60 g.) was treated with a suspension of $\text{Ca}(\text{OH})_2$ (25 g.) in water (500 ml.) at 20° for 2 weeks, 2 months and 6 months. The mixtures were stirred from time to time. The reactions were carried out in sealed Le Meilleur preserving jars. No special precautions were used to exclude CO_2 and pH 12.6 was maintained throughout.

Sodium hydroxide solution. Collagen (60 g.) was treated with 20% Na_2SO_4 solution (375 ml.) for 1 day at 20°. The liquor was poured off and used to dissolve NaOH (11 g.), then re-introduced to the collagen for 2 days, 5 days, 12 days and also 10 days with 19 g. of NaOH.

Sulphuric acid (hot). The process used (Deutsches Gelatine Fabriken, 1952) was to soak collagen (60 g.) in 20% Na_2SO_4 solution (300 ml.) containing conc. H_2SO_4 (3.2 ml.) for 24 hr. The mixture was then heated at 80° for 30 min. and also 40 min.

Dual-soak process. Three main variables were examined: strength of HCl suitable for ossein, length of acid treatment, reversal of the procedure by treating first with saturated $\text{Ca}(\text{OH})_2$; all at 20°. The collagen was washed between each treatment: 3 days in 2.5N-HCl, then 9 days in $\text{Ca}(\text{OH})_2$; 5 days in 2.5N-HCl, then 9 days in $\text{Ca}(\text{OH})_2$; 7 days in 2.5N-HCl, then 9 days in $\text{Ca}(\text{OH})_2$; 2 days in 1.67N-HCl, then 13 days in $\text{Ca}(\text{OH})_2$; 3 days in 1.67N-HCl, then 13 days in $\text{Ca}(\text{OH})_2$; 7 days in $\text{Ca}(\text{OH})_2$, then 3 days in N-HCl.

Mucoprotein estimations. The extent of removal of mucoproteins from collagen by alkaline and acid solutions was examined by the method described in Courts (1959) based on the diphenylamine reaction of Dische (1929). The collagen was first enzymically digested by one of two methods.

(i) Disintegrated collagen (1 g. dry wt.) was covered with water (20 ml.) and held at about 70° for 15 min. After cooling to about 40°, a 0.4% solution of crystalline trypsin (Armour Laboratories; 1 ml.) was added, and the whole held at 37° for 1 day.

(ii) Disintegrated collagen (1 g. dry wt.) was treated at 37° with 20 ml. of solution prepared in the following way: crude papain (Norman Evans and Rais Ltd., Stockport, Cheshire; 5 g.) was shaken for 1 hr. in water (500 ml.) containing 5N-HCl (1 ml.), and filtered. Into the filtrate was dissolved urea (90 g.) and mercaptoacetic acid (5 ml.), and the solution was adjusted to about pH 5.5. Crude papain is heavily contaminated with mucoprotein and a blank on this solution was necessary.

Aqueous solutions of certain protein/polysaccharide complexes are partitioned in contact with a solution of

phenol saturated with water at 20°; the phenol dissolves the protein and the polysaccharide is retained in the aqueous phase. A solution of phenol was prepared by dissolving 100 g. of phenol in 100 ml. of water at about 50° and allowing this to stand overnight at 20°. The upper layer was discarded.

The digests obtained were carefully shaken with the solution of phenol at 20° and the colorimetric determinations carried out as described for gelatin by Courts (1959). In estimating mucoprotein in the reaction liquors, these were first neutralized. Those containing Na_2SO_4 were allowed to stand at 4° overnight to allow this salt to crystallize. The supernatant liquors were then shaken with an equal volume of the phenol solution at 20° and the estimation was continued as before. In certain instances the increased density of the sulphated liquor causes the aqueous phase to appear below the phenolic phase.

Shrinkage temperature. Collagen fibres undergo a striking axial contraction on heating, often shrinking to 30% of the original length at approximately constant volume. A full account of shrinkage phenomena and measurements is given by Gustavson (1956). When collagen fibres are in equilibrium with water the temperature of shrinkage (T_s) may be adequately defined within 2° or 3°, although the phenomenon is rarely sharp. Flory & Garrett (1958) have shown that the transition occurring during collagen shrinkage involves the melting of crystalline regions. W. E. Braybrooks (personal communication) showed that T_s for sheepskin is reduced by the action of saturated $\text{Ca}(\text{OH})_2$.

In the present work, T_s was measured on neutral pieces of collagen–water or eucollagen–water systems freely suspended in toluene. After warming to 35°, the temperature was raised by 1°/min. It was found initially that the shrinkage of highly swollen collagen or eucollagen suspended in water was less sharply defined than with native collagen. These materials, however, have a considerable bound-water environment. Thus if the heating is carried out in a water-insoluble liquid, such as toluene, the shrinkage is more readily observed on account of the forcing out of drops of interstitial water or, with eucollagen, the melting into gelatin. The T_s values for swollen materials suspended in toluene are the same as when suspended in water, within the limits of the experimental procedure.

Swelling factor. The weight ratio (collagen + water)/collagen measured at about pH 7 after a given treatment is called the swelling factor. It is evaluated by weighing the washed material, after allowing it to drain for 5 min., and dividing the weight by the weight of dry collagen which it contains (dried to constant weight at 105°).

RESULTS

Following the definition of eucollagen given earlier, when collagen is partially converted into eucollagen the extent of the reaction may be indicated by the yield of gelatin obtained with standard thermal conditions (2 hr. at 60°). During this time, little additional hydrolysis of collagen occurs, so that the yield consists of gelatin produced solely by a configurational change in the eucollagen present at the start of extraction. This yield is given in

Table 1. Sodium salicylate (2M) gives a similar yield at 18°, 24 hr. (A. Courts, unpublished work). The rate of reaction with cold alkali is, as expected, much greater at about pH 14 than at pH 12.6. The prior soak of the collagen in acid has a marked effect on subsequent reactions at pH 12.6 and the rates then simulate those at the higher pH values without prior acid treatment.

The changes in the mucoprotein content of collagen with the selected treatments are shown in Table 4. This emphasizes the effect of alkalis in removing mucoprotein from collagenous tissue, but shows also that strong acids may remove substantial quantities. Thus cold 2.5N-HCl extracts

Yields are based on treated collagen. The gelatin yield may be regarded as the proportion of eu collagen developed by the treatment. For conditions, see text.

about 90% of available mucoproteins and 1.67N-HCl about 75%. The acid process at 80°, which uses 0.2N-H₂SO₄, extracts up to 60% of the mucoprotein into the reagent. Table 4 indicates the mucoprotein content of the ossein to be about 2.2%. After the alkali or acid treatments, the collagen was given a preliminary wash in 1 l. of water and then washed to neutrality in running water. It is evident that this continuous washing removes substantial proportions of the mucoprotein after the treatment with NaOH.

The degree of swelling in collagen, which has been modified by the reagents used, is shown in Table 5. Collagen treated with 1.67N-HCl has a swelling factor of 3.5 after 2 and 3 days. This value was not altered by the subsequent treatments with Ca(OH)₂. There is no overall indication that the swelling factor is connected with eucollagen formation, although there is an apparent relationship within each alkaline series. The fall in shrinkage temperature of ossein from 67° to the values shown in Table 3 suggests a minimum value of 44° attained by either acid or alkali.

DISCUSSION

The conversion of collagen into eucollagen involves the progressive hydrolysis of especially labile peptide bonds. Where the cleavage is by alkali, a minimum number-average chain weight (C_n) of 60 000–65 000 is attained and in any subsequent transition to gelatin this value is not greatly modified, if secondary thermal hydrolysis after solution of the gelatin is avoided. This implies that the transition, eucollagen to gelatin, does not require further covalent-bond breaking along the peptide chains, and in Courts (1958) the view is put that the stability of eucollagen relies mainly on regions of strong hydrogen-bonding. Gelatin may therefore be described as the molecularly disorientated form of eucollagen, although partial re-orientation may occur during gelation and also in the dry gelatin film (Robinson, 1953).

The principal *N*-terminal residue of bone collagen after demineralizing with acid is glycine, but the amino groups of serine, threonine, aspartic acid and glutamic acid and alanine appear after only mild alkaline treatment, and develop with time or intensity of treatment to a maximum total of about 1.6 residues/100 000 g. of degraded collagen, corresponding to C_n 62 000. It has not been found possible to increase this *N*-terminal residue total in degraded collagen, either by prolonging the alkaline reaction or by heating in water at 93°. It is apparent that the existence of collagen molecules in the insoluble form with C_n below 62 000 is only brief. Where C_n is reduced below this value by cold reagents, then solution into the reagent occurs.

Table 2. *N*-Terminal amino acid residues of treated collagen and derived gelatins

Residues are expressed as moles/100 kg. of protein. C_n is number-average chain weight calculated from total. For conditions, see text. The times of treatment are given in the column headings.

(a) *N*-Terminal residues released by treatment with Ca(OH)₂.

	2 weeks						2 months						6 months					
	Collagen			Gelatin			Collagen			Gelatin			Collagen			Gelatin		
	Unheated	60°	80°	60°	80°	93°	Unheated	60°	80°	60°	80°	80°	Unheated	60°	80°	60°	80°	80°
	107	93	94	39	2.56	1.75	64	69	64	51	43	69	69	50	58	69	50	58
Glycine	0.40	0.46	0.49	1.40	0.90	1.19	0.76	0.72	0.77	1.01	1.13	0.66	0.66	1.00	0.83	0.09	0.13	0.20
Serine	0.22	0.26	0.23	0.31	0.24	0.29	0.34	0.26	0.29	0.29	0.30	0.19	0.19	0.20	0.16	0.05	0.13	1.76
Threonine	0.10	0.10	0.09	0.21	0.14	0.13	0.10	0.12	0.13	0.16	0.21	0.18	0.18	0.15	0.18	0.12	0.12	0.08
Alanine	0.05	0.07	0.06	0.13	0.11	0.19	0.10	0.10	0.10	0.12	0.13	0.12	0.12	0.12	0.08	0.15	0.19	0.14
Aspartic acid	0.06	0.08	0.07	0.11	0.09	0.16	0.14	0.10	0.13	0.08	0.14	0.15	0.15	0.19	0.14	0.09	0.21	0.17
Glutamic acid	0.06	0.06	0.07	0.30	0.13	0.20	0.08	0.09	0.08	0.17	0.29	0.09	0.09	0.21	0.17	0.05	0.13	0.20
Others	0.04	0.04	0.05	0.10	0.06	0.10	0.05	0.06	0.06	0.15	0.13	0.05	0.05	0.13	0.20	0.05	0.13	0.20
Total	0.93	1.07	1.06	2.56	1.75	2.26	1.57	1.45	1.56	1.98	2.33	1.44	1.44	2.00	1.76	1.44	2.00	1.76
10 ⁻³ C_n	107	93	94	39	2.56	1.75	64	69	64	51	43	69	69	50	58	69	50	58

Table 2 (cont.)
(b) N-Terminal residues released by treatment with NaOH.

Concn. of NaOH and time of treatment	3% (w/v); 2 days		3% (w/v); 5 days				3% (w/v); 12 days		5% (w/v); 10 days			
	Collagen		Collagen		Gelatin		Collagen unheated		Collagen Unheated		Gelatin 60°	
	Unheated	60°	Unheated	60°	80°	93°	Unheated	60°	Unheated	60°	Gelatin 60°	60°
Glycine	0.49	0.44	0.65	0.58	0.66	1.02	1.00	0.83	0.75	0.78	1.13	1.13
Serine	0.20	0.19	0.22	0.24	0.22	0.23	0.20	0.20	0.19	0.20	0.30	0.30
Threonine	0.09	0.01	0.09	0.11	0.08	0.10	0.09	0.12	0.16	0.10	0.22	0.22
Alanine	0.07	0.06	0.10	0.12	0.11	0.10	0.17	0.11	0.12	0.14	0.18	0.18
Aspartic acid	0.08	0.09	0.09	0.11	0.09	0.10	0.12	0.12	0.12	0.19	0.16	0.16
Glutamic acid	0.08	0.06	0.09	0.08	0.06	0.13	0.10	0.18	0.07	0.06	0.16	0.16
Others	0.04	0.05	0.03	0.04	0.06	0.10	0.13	0.10	0.10	0.10	0.15	0.15
Total	1.05	0.96	1.26	1.28	1.28	1.78	1.81	1.66	1.51	1.57	2.30	2.30
10 ⁻³ C _n	95	104	79	78	78	56	55	60	66	64	44	44

(c) N-Terminal residues released by treatment with H₂SO₄.

	30 min.				40 min.							
	Collagen		Gelatin		Collagen		Gelatin		Collagen		Gelatin	
	Unheated	60°	80°	93°	Unheated	60°	80°	93°	Unheated	60°	80°	93°
Glycine	0.33	0.61	0.80	1.39	0.73	1.39	1.69	1.73	0.96	0.96	1.50	1.73
Serine	0.07	0.14	0.12	0.13	0.11	0.13	0.15	0.17	0.11	0.11	0.14	0.17
Threonine	0.09	0.12	0.07	0.06	0.08	0.06	0.09	0.09	0.07	0.07	0.08	0.09
Alanine	0.07	0.13	0.26	0.39	0.15	0.39	0.59	0.67	0.16	0.16	0.39	0.67
Aspartic acid	0.05	0.05	0.17	0.12	0.07	0.12	0.16	0.18	0.07	0.07	0.14	0.18
Glutamic acid	0.12	0.21	0.07	0.09	0.09	0.09	0.10	0.08	0.11	0.11	0.07	0.08
Others	0.05	0.15	0.07	0.05	0.04	0.05	0.12	0.10	0.10	0.10	0.05	0.10
Total	0.78	1.43	1.56	2.23	1.27	2.23	2.89	3.03	1.58	1.58	2.37	3.03
10 ⁻³ C _n	128	70	64	45	78	45	35	65	64	43	33	33

(This probably accounts for the loss of collagenous material which occurs in very acid or alkaline solutions.) Collagen molecules thermally degraded above the shrinkage temperature to C_n below 62 000 are likely to go into solution as gelatin. Whatever the C_n value attained by degrading

collagen with alkali, this value does not alter as a result of subsequent thermal conditions applied to it, even though the gelatin formed in the process may become thermally degraded and hence show a further development of terminal amino groups.

Table 3. *Changes in N-terminal amino acid residues and shrinkage temperature of collagen*

Residues are expressed as moles/100 kg. of protein. C_n is number-average chain weight calculated from total. N-Terminal residues were released by treatment with 2.5N-HCl, then $\text{Ca}(\text{OH})_2$, for 9 days. For conditions, see text.

	After 2.5N- HCl (3 days)	After 2.5N- HCl and then $\text{Ca}(\text{OH})_2$	Diff.	After 2.5N- HCl (5 days)	After 2.5N- HCl and then $\text{Ca}(\text{OH})_2$	Diff.	After 2.5N- HCl (7 days)	After 2.5N- HCl and then $\text{Ca}(\text{OH})_2$	Diff.
Glycine	0.39	0.64	+0.25	0.42	0.70	+0.28	0.48	0.80	+0.32
Serine	0.10	0.24	+0.14	0.10	0.27	+0.17	0.15	0.27	+0.12
Threonine	0.18	0.17	-0.01	0.24	0.18	-0.06	0.34	0.25	-0.09
Alanine	0.13	0.10	-0.03	0.15	0.07	-0.08	0.18	0.12	-0.06
Aspartic acid	0.08	0.14	+0.06	0.08	0.15	+0.07	0.16	0.26	+0.10
Glutamic acid	0.05	0.09	+0.04	0.06	0.07	+0.01	0.08	0.08	—
Others	0.10	0.07	—	0.12	0.08	—	0.17	0.08	—
Total	1.03	1.45	—	1.17	1.52	—	1.56	1.86	—
$10^{-3}C_n$	98	69	—	85	66	—	64	54	—
T_g	52°	44°	—	48°	46°	—	45°	45°	—

Table 4. *Mucoprotein content of untreated and treated collagen and reagents*

For conditions of the treatments, see text. Values are given as g. of mucoprotein per 100 g. of collagen.

	Un- treated	Treatment with $\text{Ca}(\text{OH})_2$			Treatment with NaOH 3% (w/v)				Treatment with H_2SO_4 (40 min.)
		2 weeks	2 months	6 months	2 days	5 days	12 days	5% (w/v) 10 days	
Collagen	2.15	1.10	0.42	0.00	0.40	0.25	0.15	0.08	0.87
Reagent	—	0.87	1.32	1.20	0.20	0.50	0.85	1.80	1.44
First wash	—	0.00	0.27	1.03	0.63	0.60	0.33	0.37	0.01
Total	2.15	1.97	2.01	2.23	1.23	1.35	1.33	2.25	2.32
Dual soak									
Treatment with 2.5N-HCl					Treatment with 1.67N-HCl		2 days	3 days	
3 days	5 days	7 days			Treatment of residual collagen for 13 days with $\text{Ca}(\text{OH})_2$		1.70	1.75	
2.05	2.05	2.07					0.30	0.20	

Table 5. *Swelling factors of treated collagen*

For conditions, see text.

Treatment with $\text{Ca}(\text{OH})_2$			Treatment with NaOH 3% (w/v)			Treatment with H_2SO_4	
2 weeks	2 months	6 months	5 days	12 days	5% (w/v) (10 days)	30 min.	40 min.
2.0	2.2	5.0	2.3	2.6	2.7	1.8	1.8
Treatment with 2.5N-HCl and then 9 days with $\text{Ca}(\text{OH})_2$			Treatment with 1.67N-HCl and then 9 days with $\text{Ca}(\text{OH})_2$		Treatment for 7 days with $\text{Ca}(\text{OH})_2$ and then 7 days with 1.67N-HCl		
3 days	5 days	7 days	2 days	3 days			
5.1	6.0	10	3.5	3.5			3.5

The action of hydroxyl ions on the peptide bonds of collagen is the same whether calcium hydroxide or sodium hydroxide is the source, although there is an apparent correlation between the rate and the hydroxyl ion concentration. There is, however, a second factor to which extremes of alkalinity and acidity contribute in an important way. A preliminary soak of collagen in a strong cold acid accelerates the subsequent conversion into eucollagen with calcium hydroxide, by a factor as high as 20-fold. The effect is probably best seen by comparing, as in Table 6, the dual soak experiment which included a prior 3-days treatment in 2.5N-hydrochloric acid with the experiment in which calcium hydroxide alone was used.

It is clear from Table 1 that, in order to accelerate the change from collagen into eucollagen, the order of use of the reagents must be first acid, then calcium hydroxide. The reverse order is ineffective. It is therefore possible that the difference between pretreatment with calcium hydroxide and sodium hydroxide, where the latter gives a much higher hydroxyl ion concentration, does not arise from the rate difference of a single type of hydroxyl ion-catalysed peptide-bond hydrolysis. The possibility exists that, in the early stages, the high alkalinity performs the same initial function as high acidity in leaving the partially degraded collagen in a state which then allows the alkaline hydrolysis of peptide bonds to proceed more rapidly. The modification needed to permit a rapid reduction in chain-size is not, as yet, fully understood. Some insight, however, may be given by the production of *N*-terminal residues by acids in the cold (Table 3,

first stage of the dual soak): (a) the *N*-terminal residues have developed to the same extent as in the presence of strong alkali, with the exception of *N*-terminal glycine and serine; (b) the collagen chains have decreased in size to C_n values resembling high-grade gelatin obtained through acid treatment, namely, 65 000–70 000; (c) the shrinkage temperature (T_s) values (Table 3) would indicate, by analogy with the collagen-alkali system, extensive eucollagen formation (the shrinkage temperatures are only slightly altered by the subsequent eucollagen formation in calcium hydroxide). Yet results based on gelatin formation (e.g. at 60°; 2 hr.) show that the conversion of collagen into eucollagen is not brought about in the cold by hydrogen ions alone. The T_s value is therefore misleading as a guide to the extent of eucollagen formation under acid conditions, although it is clear that the collagen stability must become greatly impaired.

It is reasonable to assume that the formation of eucollagen is in some way allied to the increase of *N*-terminal glycine and serine so that some additional peptide bonds require to be broken after the changes induced by acid have taken place.

The hot-acid treatment in the presence of sodium sulphate gives a similar impression of labile peptide bonds leading to *N*-terminal glycine. Unlike the wholly alkaline reactions, the C_n of this acid-treated collagen changes during the transition to gelatin. Thus the value C_n 130 000 is halved during extraction, which takes place after the sodium sulphate has been washed out. This change is almost entirely due to the development of

Table 6. *Effect of acid treatment on the alkaline hydrolysis of collagen*

2.5N-HCl treatment (days)	Ca(OH) ₂ treatment (days)	Eucollagen* content (%)	Collagen lost into Ca(OH) ₂ (%)	Swelling factor	<i>N</i> -Terminal glycine†	C_n
0	180	53	47	5.0	0.64	69 000
3	9	48	50	5.1	0.66	69 000

* Based on gelatin yields (Table 1).

† Moles/100 kg. of collagen.

Table 7. *Thermal effects on the C_n of collagen and gelatin*

fresh *N*-terminal glycine. It is clear that the sodium sulphate had previously protected hydrolysis at this peptide bond. The thermal effects on C_n are summarized in Table 7.

The quantity of mucoprotein in ossein is about 2.2% of the organic matter. The discrepancy between this value and the value 0.8% given by Eastoe & Eastoe (1954) may be due to the different bone tissue involved and to different demineralization conditions. Since the mucoprotein content of ox bone was found to be about 5.5% of the organic matter (Courts, 1959), it appears that about 60% was removed in the acid demineralization of bone.

The distribution of mucoprotein in collagen-acid and collagen-alkali systems has not provided conclusive views on the function of the polysaccharide portion in stabilizing collagen at the molecular level. On the whole, the evidence is against any stabilizing action. It has been argued (Jackson, 1953) that those reagents which extract mucoprotein from collagen also impair its stability, as shown by fall in T_g values. It is possible to extract a substantial part of the mucoprotein from ossein with 5% sodium dodecyl sulphate. This treatment does not alter the rate of alkaline pretreatment of the collagen. This, together with the fact that calcium chloride solution can be used for extracting mucoprotein, emphasizes the non-covalent nature of its association with collagen. If the extent of eucollagen formation may be used as an index of reduced collagen stability, Table 8 then shows that mucoprotein content is not necessarily correlated with collagen stability. There are also the gross observations that those connective tissues with the largest proportions of mucoprotein appear to have the least stability towards acids and alkalis. Thus the mucoprotein proportion in young mammalian tissue is greater than in the corresponding adult tissue (Sobel & Marmorsten, 1956; Stidworthy, Masters & Shetlar, 1958), yet the stability of the adult tissue is greater (Kohn & Rollerson, 1958). Ossein, with higher mucoprotein content (Courts, 1959), is easier to hydrolyse to eucollagen than skin or tendon (Courts & Stainsby, in preparation). The collagenous connective tissue of rabbit, substantially soluble in dilute acid buffers (Plotnikova, 1947), has a high mucoprotein content compared with cattle connective tissue (Courts, 1959), which shows only very limited solubility.

Table 8. *Mucoprotein and eucollagen contents of some pretreated collagens*

Pretreatment	Mucoprotein left with collagen (%)	Eucollagen content (%)
NaOH, 3%; 2 days	19	5
Ca(OH) ₂ ; 2 months	18	32
H ₂ SO ₄ ; 40 min., 80°	40	35

Although these observations set out to show that that major portion of the mucoprotein present in connective tissue does not play any part in collagen stability at the molecular level, very small quantities, which are not removed by neutral reagents, may still be of importance.

SUMMARY

1. Alterations in the structure of collagen by alkali and acid have been assessed by analysis of the *N*-terminal residues developed.

2. In its least stable form, the collagen fibrillar configuration is unstable in water at 60°, transforming into gelatin in a few minutes. This form of collagen is termed 'eucollagen'. It is suggested that eucollagen relies almost entirely on regions of strong hydrogen-bonding for its molecular stability.

3. The structure of bone collagen is considered to involve chains built from subunits each with number-average chain weight between 60 000 and 70 000. Chain-weight values below 60 000 are not generally encountered for eucollagen.

4. Although hydrochloric acid (1.0-2.5*N*) does not convert collagen into eucollagen, it can modify the collagen to permit a rapid acceleration of the reaction at pH 12.6.

5. The solution of mucoprotein in acid as well as alkali is noted. The bulk of the mucoprotein present in ossein, which is about 2.2%, is not likely to be a major factor in collagen stability.

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Chemical Changes in Skeletal Muscle During Development

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Skeletal muscle has been investigated from many angles, and certain aspects of the effect of development on its composition have received a good deal of attention. It is well known, for instance, that during development the percentage of water in skeletal muscle falls (Needham, 1931; Yannet & Darrow, 1938; Hines & Knowlton, 1939; McMeekan, 1940; McCance & Widdowson, 1956*a*) and that this change is accompanied by a fall in the concentrations of the extracellular ions sodium and chloride. At the same time there is an increase in the percentage of protein (Needham, 1931; McCance & Widdowson, 1956*a*) and of the intracellular constituents potassium and phosphorus. These changes show that development is associated with a decrease in the percentage of extracellular fluid and an increase in the 'cell mass'. It is not known if there is any change in the composition of the muscle cell with development, and the present study was undertaken with this objective in view.

Muscle cells cannot be separated for direct analysis, and their composition must be determined indirectly. McCance & Widdowson (1956*a*) have shown that the amount of nitrogen per unit weight of potassium in skeletal muscle increased with development and have interpreted this as possibly indicating a change in the gross composition of the muscle cell. There are, however, two sources of uncertainty about this. First, it has been suggested that more of the potassium is outside the cells in the foetus than in the adult (Widdowson & McCance, 1956) and, secondly, that changes in the proportion of the extracellular proteins collagen, elastin and reticulin may take place during development. Robinson (1952*a*) has shown that such changes do in fact take place in the muscle of the developing chick.

Little information appears to be available about the changes in the proportion of different proteins

in developing mammalian muscle. Hermann & Nicholas (1948) separated a number of protein fractions from the muscle of the rat at various ages, but the method of separation used by these workers did not effect a complete separation of the intracellular from the extracellular proteins. The method devised by Robinson (1952*a*) did effect such a separation, and at the same time enabled the intracellular proteins to be separated into two groups consisting of the sarcoplasmic and fibrillar proteins. This method has now been applied to the muscle of man and the pig at various stages of development in order to ascertain if there is any change in the protein composition of the cell during this process, and at the same time to provide a more reliable basis of reference for other intracellular substances.

EXPERIMENTAL

Materials

Human. Thigh muscle was obtained from four foetuses of approx. 14 weeks' gestation, weighing about 30 g., and from four foetuses of 18–24 weeks' gestation, weighing between 200 and 300 g. The foetal age was calculated from the crown–heel length by means of the formula derived by Scammon & Calkins (1929). The foetuses were all obtained at hysterotomy, pregnancy being terminated for various reasons. Thigh muscle was also obtained from four full-term newborn babies which failed to respond to resuscitation, three babies aged 4–7 months who were suffocated in their cots, one 11-year-old boy and one 16-year-old boy and from four men who were killed in street accidents. The ages of the men were 21, 27, 40 and 43 years.

The foetuses were brought to the Laboratory, generally still in their membranes, and they were dissected within an hour of delivery. Muscle was taken from the seven babies, the two boys and the four adults at autopsy, the bodies being stored in a refrigerator meanwhile.

Pig. The pigs came from the Laboratory farm. The stock was originally Large White × Essex cross, subsequently