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The Effect of Alkalis on Collagen

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An essential process in the conversion of hides and skins to most types of leather is the removal of the hair or wool by treatment in suspensions of lime and sodium sulphide, usually in the cold, for periods varying from a few hours to several weeks.

Although several workers have investigated the effect of mild alkaline treatment on collagen (Marriott, 1928, 1931; Küntzel & Phillips, 1933; Kubelka & Knöedel, 1938; Highberger & Stecker, 1941; Ames, 1944), each has dealt only with one or two of the possible changes which may occur, and in only one instance (Marriott, 1931) has a collagen been used which has had no previous alkaline or enzymic treatment.

The present investigation has been carried out, therefore, on a collagen which has received no previous chemical treatment likely to cause chemical modification (see Bowes & Kenten, 1948), with the aim of obtaining more precise information regarding possible changes occurring during mild alkaline treatment.

EXPERIMENTAL

Alkali treatment of collagen. Collagen prepared as previously described (Bowes & Kenten, 1948) was cut into cubes about 1 cm. square, and 100 g. of this material, together with 310 ml. of water, 1.55 g. of $\text{Ca}(\text{OH})_2$, and 90 ml. of N-NaOH were incubated in a sealed flask at 20° . A preliminary experiment showed that these quantities gave an initial OH^- concentration of 135 m-equiv./l. (pH 13.3 at 20°), and a final value of 68 m-equiv./l. (pH 13.0 at 20°). After 14 days the flask was cautiously opened to avoid loss of NH_3 , sufficient HCl added to bring the contents to pH 4.5–5.0, the flask resealed and left at 20° for 3 days. Two batches of collagen (I and II) were treated in this way.

The collagen was filtered off, washed in running water for several hours, treated repeatedly with acetic acid pH 3 containing 1.5% NaCl , exhaustively washed with water at pH 8, finally with water at pH 6, then dehydrated with acetone. The pH of the last wash water was 5.5. (A little toluene was added to all wash waters to inhibit the growth of micro-organisms.)

An appreciable amount of protein matter (solubilized collagen) was precipitated on neutralization. This material was filtered off with the collagen and removed during the washing in running water.

The total N, amino N, NH_3 and urea were determined in the solution in which the collagen had been treated. It was assumed that the solution held by the collagen was of the same composition as the external solution, and that the total volume was equal to that of the solutions added plus the water held by the original collagen, and the results were calculated accordingly. The total N, amino N, amide N, arginine, total hydroxy acids (serine + threonine + hydroxylysine), and the titration and swelling curves of the collagen, were also determined. All results are expressed on moisture- and ash-free collagen. Since the total N of the alkali-treated collagen is lower than that of the original collagen, the results expressed as a percentage of the total N may be misleading, and they have therefore been recorded also as mmol./g. protein.

Analysis. Total N, amide N and amino N were determined as previously described (Bowes & Kenten, 1948). Arginine was determined by precipitation with flavianic acid (Vickery, 1940). The value obtained (8.3%) was rather lower than that of 8.8% found for the same collagen sample by Macpherson (1946), who used a colorimetric method following the separation of the basic amino-acids by electro-dialysis.

Hydroxyamino-acids. The total hydroxyamino-acids (serine + threonine + hydroxylysine) were determined by the periodate method as described by Rees (1946), except that the periodate oxidation and subsequent distillation of the NH_3 were carried out in a micro-Kjeldahl apparatus. The following solutions were successively added to the micro-Kjeldahl apparatus, while preserving a liquid seal at the ground-glass stopper: 5 ml. of hydrolysate, 5N-NaOH to adjust the pH to 7, N- H_2SO_4 (sufficient to give a final pH of 7.0 after addition of other reagents), 1.5 ml. of NaAsO_2 solution, 6 ml. of phosphate buffer pH 7.2, 1 ml. of 0.5M- HIO_4 , 3 ml. water. The contents of the micro-Kjeldahl flask were thoroughly mixed after each addition by warming the steam flask, and thereby causing a stream of air bubbles to pass through the solution. The NH_3 (preformed and periodate) was distilled off from 5 ml. of borate buffer pH 12.0. The preformed NH_3 was determined by a separate dis-

tillation of 5 ml. of hydrolysate at pH 7.0 from 5 ml. of borate buffer.

Other determinations. Ammonia in solution was determined by addition of excess K_2CO_3 and aerating into standard acid; urea by conversion to NH_3 by the action of urease followed by the same procedure (Van Slyke & Cullen, 1914). The urea NH_3 was then calculated by difference. It was necessary to use twice as much urease as that employed by Van Slyke & Cullen (1914) in order to obtain satisfactory results; this was probably due to inhibition of the urease by the high salt concentration of the solution (cf. Conway, 1947). The amount of urea was small compared with that of NH_3 , and tests showed that under the present experimental conditions the method gave an accuracy of $\pm 10\%$.

Titration and swelling curves. The titration curve of the alkali-treated collagen (batch I) was determined in the presence of 0.5M-NaCl as previously described (Bowes & Kenten, 1948). For the swelling curves, 0.5 g. samples of air-dry collagen were placed in 100 ml. of solution containing varying amounts of HCl and NaOH at 20°. After 3 days, a sample of liquid was withdrawn for pH determination, and the pieces of collagen lightly blotted and weighed. The vessels used were the same as those employed for the titration curve, and the same precautions were taken to avoid contamination with CO_2 . The water content, expressed as a percentage of the moisture and ash-free collagen, was plotted against the final pH value of the solution.

RESULTS

Analytical

There is good agreement between the results on the two separate batches of treated collagen, indicating that the results obtained are reproducible (see Table 1).

Although the treatment given was comparatively drastic, the collagen only decreased in weight by about 5%, and was unchanged in appearance. Approximately half the amide N was liberated during 14 days' treatment, and a corresponding amount of ammonia was found in the solution; there

Table 1. *Analyses of collagen, alkali-treated collagen and of collagen dissolved by alkaline solution*

	Collagen						Solution		
	Un- treated	Alkali- treated		Increase or decrease					
		Batch I	Batch II	Batch I	Batch II	Batch I	Batch II		
Ash (%)	0.03	0.01	0.10	—	—	—	—	—	
Water (%)	22.3	23.7	23.8	—	—	—	—	—	
Total N (%)	18.6	18.2	18.1	-0.4	-0.5	Total N (mg./g.)	9.0	8.1	
Amino N (as % TPN)	2.48	2.77	2.94	—	—	Amino N (mg./g.)	4.3	4.3	
Amino N (mmol./g.)	0.33	0.36	0.38	+0.03	+0.05	—	—	—	
Amide N (as % TPN)	3.54	1.69	1.85	—	—	Ammonia N (mg./g.)	3.46	3.36	
Amide N (mmol./g.)	0.47	0.22	0.24	-0.25	-0.23	Ammonia N (mmol./g.)	0.25	0.26	
Arginine N (as % TPN)	14.45	14.17	14.24	—	—	Urea N (mg./g.)	0.45	0.42	
Arginine N (mmol./g.)	0.48	0.46	0.46	-0.02	-0.02	Urea (mmol./g.)	0.016	0.015	
Periodate NH ₃ (as % TPN)	4.54	4.45	—	—	—	—	—	—	
Periodate NH ₃ (mmol./g.)	0.60	0.58	—	-0.02	—	—	—	—	

TPN = total protein nitrogen.

was no indication that an appreciable amount of ammonia was derived from any other source. The amount of ammonia formed was almost identical with the maximum value obtained by Highberger & Stecker (1941). Further treatment for 7 days under similar conditions, however, lowered the amide N by another 0.08 mmol. to 0.16 mmol./g., with the production of 0.06 mmol. of ammonia.

The small decrease in the arginine content is less than that found by Theis & Jacoby (1941) in similar experiments, and the urea found in the solution was also less than that found by Highberger & Stecker (1941) under corresponding conditions. It appears probable that the urea arises from conversion of the arginine to ornithine residues (cf. Hellermann & Stock, 1938; Warner, 1942a); the decrease in arginine

(this small increase could be accounted for by the conversion of arginine to ornithine), and, even allowing for the fact that because of the high proline and hydroxyproline content of collagen one out of every four peptide links involves an imino and not an amino group, the alkaline treatment has apparently caused little breakdown of the polypeptide chains of the remaining insoluble collagen.

Titration curves

At pH values between 5 and 10, a longer time was required to reach equilibrium with alkali-treated collagen than with the original collagen. There was rather more soluble nitrogen at high pH values, and rather less at low pH values than with the original collagen (see Table 2).

Table 2. *Nitrogen in solution at different pH values after steeping for 3 days*

(Results expressed as mg. N/g. moisture- and ash-free collagen.)

Collagen				Alkali-treated collagen			
pH (20°)	Soluble N	pH (20°)	Soluble N	pH (20°)	Soluble N	pH (20°)	Soluble N
1.16	0.85	12.86	1.44	1.18	0.48	—	—
1.32	0.77	12.71	1.08	—	—	12.77	2.15
1.51	0.69	12.66	1.21	1.42	0.36	12.69	2.05
1.64	0.70	12.59	1.28	1.82	0.34	12.54	2.26
—	—	12.46	1.04	—	—	12.39	1.26
—	—	—	—	—	—	12.20	1.45

content, however, is rather greater than can be accounted for as urea, indicating some other type of breakdown. The alkali-treated collagen, when tested for the presence of citrulline by the method of Fearon (1939), gave a very faint pink colour, the original none at all.

Within experimental error, the ammonia formed by the action of periodate on the untreated and alkali-treated collagen is the same, indicating a negligible destruction of the hydroxyamino-acids by the alkaline treatment. The hydroxyamino-acids of the collagen used in this investigation have also been determined by Rees (1946), who obtained a value of 4.57% for the periodate NH_3 (expressed as a percentage of the total protein N), in good agreement with the present determination of 4.54%.

The ammonia and urea in the solution account only for about half of the total N present. The figure for the amino N of the solution is likely to be too high owing to the presence of ammonia (25–30% of which may be returned as amino N (Warner, 1942b; Frost & Heinsen, 1945)), but the results suggest that this soluble N must be present as amino-acids or small peptides. It may be observed that the sum of the ammonia and urea N in solution is approximately equal to the decrease in the total N of the collagen.

The amino N of the alkali-treated collagen was only slightly higher than that of the original collagen

The titration curve of the alkali-treated collagen differs in a number of respects from the curve of the original collagen, especially in the alkaline range (see Fig. 1). The isoelectric point is shifted from pH 6.7 to 5, the acid binding is slightly increased, and on the alkaline side of the isoelectric point there is a large increase in the number of groups titrating between pH 5 and 9, and a corresponding increase in the base binding capacity. The large increase in the groups titrating between pH 5 and 9 may be attributed to an increase in the number of carboxyl groups arising from hydrolysis of amide groups.

The curve was analyzed in a manner similar to that of the untreated collagen (see Bowes & Kenten, 1948). It can be divided into four portions, from pH 1.5 to 5, from 5 to 7, from 7 to 9.6 and from 9.6 to 12, and to these different portions are assigned the groups shown in Table 3. Imino groups, due to breakdown of peptide links involving proline or hydroxyproline, are taken as mainly titrating between pH 7 and 9.6.

Assuming that the imidazole groups are unaffected by alkaline treatment (Plimmer, 1916), and that they titrate in the same range as in the untreated collagen, it can be deduced that there are 0.14 mmol. uncharged carboxyl groups/g., and 0.13 mmol. basic groups/g. (α -amino, imidazole or imino), titrating between the isoelectric point and pH 9.6. The assumption that the imidazole groups

are not affected may not be strictly true, since there is some evidence that gelatin contains less histidine than collagen (Chibnall, 1946). The difference, however, is small, and even if a corresponding decrease in histidine does occur here, it would not materially affect the calculation. The same conclusion is

reached if the increase in acid-binding capacity is taken as due to increase in the number of α -amino and imino groups, again assuming that these groups titrate between pH 5 and 9.6, and that the α -amino and imidazole groups present in the original collagen remain unchanged.

Fig. 1. Titration curves of ox-hide collagen before (circles) and after (triangles) treatment with alkali.

Table 3. *Analyses of titration curves*

(Results expressed as mmol./g.)		Original collagen	Alkali-treated collagen
Group	Method of calculation		
(a) Charged carboxyl	Titration from pH 1.5 to 5	0.87	0.96
(b) Uncharged carboxyl + imidazole	Titration from pH 5 to 7	0.03	0.17
(c) Imidazole, α -amino + imino	Titration from pH 7 to 9.6	0.04	0.10
(d) ϵ -Amino	Titration from pH 9.6 to 12.5	0.34	0.36
(e) Uncharged carboxyl	(b) - 0.03 mmol. imidazole groups titrating in this range	0	0.14
(f) Total carboxyl	(a) + (e)	0.87	1.10
(g) α -Amino + imino	(c) - 0.02 mmol. imidazole groups titrating in this range	0.02	0.08
(h) Guanidino	(a) + (e) - (b) - (c) - (d)	0.49	0.47
(i) Amide	From analysis	0.47	0.22
(j) Dicarboxylic acids	(f) + (i) - (g)*	1.32	1.24

* It is assumed that terminal carboxyl groups are equivalent to the free α -amino and imino groups.

If these conclusions are correct, there is an apparent decrease in the dicarboxylic acids, which suggests that there may have been specific loss of these amino-acids in the fraction of the collagen rendered soluble by the alkaline treatment.

The decrease in arginine content calculated from the titration curve agrees well with that found by analysis (Table 1), and the small increase in groups titrating between pH 9.6 and 12 is in accordance with the hypothesis that the arginine is converted to ornithine (pK 10.76) (Cohn & Edsall, 1943). There is no indication of any decrease in the number of ϵ -amino groups.

Swelling curve

The uptake of water by the collagen at different pH values before and after treatment with alkali

Fig. 2. The water uptake of ox-hide collagen before (triangles) and after (circles) treatment with alkali.

is shown in Fig. 2. After exposure to the acid or alkaline solutions, the pieces of alkali-treated collagen had a roughened appearance especially at

high or low pH values. This was probably an indication of the beginning of disintegration. At all pH values the water uptake is appreciably increased by the alkaline treatment, and on the alkaline side of the isoelectric point the shape of the curve is appreciably different from that of the original collagen.

Analysis of samples of skin collagen after alkaline processing ('liming')

The total N, amino N and amide N were determined in a number of skins which had been treated commercially with calcium hydroxide and sodium sulphide to remove the hair ('limed'). The titration curves and arginine contents of three samples were also determined.

Table 4 shows that, with the exception of the sheep-skin collagens, the total N of all the samples was of the same order as that of the alkali-treated ox-hide collagen. The amide-N values varied from 0.25 to 0.47 mmol./g., the sheep skins on the whole having the lowest values. These skins had been treated with a commercial preparation of trypsin ('bating') and with sulphuric acid (approx. 1%) and sodium chloride ('pickling'), and this is probably the cause of the lower values. In a number of cases, the amino N of the commercially treated samples is lower than that of the ox-hide collagen.

The titration curves of sheep skins 1, 2, 3 differ from one another and from the curves for ox-hide collagen, especially in the pH range 6-9.

The curves are more difficult to interpret in terms of the reactive groups than those of the ox-hide collagen. The various sections of the curve, especially those due to titration of unionized carboxyl, imidazole, α -amino and imino groups tend to merge into one another. Treating the curves similarly to that of the alkali-treated collagen, the analyses given in Table 5 were deduced.

Table 4. Total N, amide N and amino N of collagen of some commercially treated skins

Source of collagen	Total N (%)	Amide N		Amino N	
		(%)	(mmol./g.)	(%)	(mmol./g.)
Ox-hide collagen (no alkaline treatment)	18.6	0.66	0.47	0.46	0.33
Sheep skins 1	17.3	0.42	0.30	—	—
2	17.1	0.42	0.30	0.59	0.42
3	17.2	0.35	0.25	0.50	0.36
4	17.4	0.50	0.36	0.45	0.32
5	17.3	0.49	0.35	0.49	0.35
Calf skins 1	18.2	0.56	0.40	—	—
2	18.2	0.52	0.37	—	—
3	18.0	0.55	0.39	0.42	0.30
Ox hide 1	18.1	0.66	0.47	0.46	0.33
2	18.1	0.42	0.30	0.45	0.32
3	18.3	0.46	0.33	0.48	0.34
4	18.1	0.57	0.41	0.42	0.30

Fig. 3. Titration curves of commercially treated sheep skins.

Table 5. Analyses of titration curves of alkali-treated sheep-skin collagens

(Results expressed as mmol./g.)			Alkali-treated sheep-skin collagen		
Group	Method of calculation		Sample 1	Sample 2	Sample 3
(a) Charged carboxyl	Titration from pH 1.5 to isoelectric point		0.89	0.97	0.88
(b) Uncharged carboxyl + imidazole	Titration from isoelectric point to pH 7		0.10	0.06	0.16
(c) Imidazole, α -amino + imino	Titration from pH 7 to 9.6		0.14	0.10	0.13
(d) ϵ -Amino	Titration from pH 9.6 to 12.5		0.34	0.32	0.26
(e) Uncharged carboxyl	(b) - 0.03 mmol. imidazole groups titrating in this range		0.07	0.03	0.13
(f) Total carboxyl	(a) + (e)		0.96	1.00	1.01
(g) α -Amino + imino	(c) - 0.02 mmol. imidazole groups titrating in this range		0.12	0.08	0.11
(h) Guanidino	(a) + (e) - (b) - (c) - (d)		0.38	0.52	0.46
(i) Amide	From analysis		0.30	0.30	0.25
(j) Dicarboxylic acids	(f) + (i) - (g)*		1.14	1.22	1.15

* It is assumed that terminal carboxyl groups are equivalent to terminal α -amino and imino groups.

DISCUSSION

Considering the comparative severity of the alkaline treatment, the chemical modification of the collagen is comparatively small. Apart from the solubilization of about 5 % of the collagen, the only reaction taking place to any appreciable extent is the hydrolysis of amide groups. Since the loss of amide groups increases with time, it appears probable that all the amide N would be liberated if the treatment were sufficiently prolonged.

The small decrease in arginine content and the presence of urea in solution indicates a breakdown to ornithine, as postulated by Hellermann & Stock (1938) and Warner (1942*a*). A positive test for citrulline in the alkali-treated collagen indicates that the conversion of arginine to citrulline and ammonia also takes place to a very small extent (see Warner, 1942*b*). The amount of ammonia arising from arginine is negligible compared with that due to deamidation.

Nicolet, Shinn & Saidel (1942) have shown that treatment of silk fibres with boiling 0.1 M-alkali for 1-2 hr. causes partial destruction of serine and threonine, producing an approximately equivalent number of amide groups. There is no evidence that such a reaction occurs even to a limited extent when collagen is treated with alkali in the cold for periods up to 14 days.

The ornithine produced by modification of the guanidino groups could account for the small increase in the base bound between pH 9.6 and 12.5, and for the greater part of the increase in free amino N. On this basis, there can be little, if any, hydrolysis of peptide links involving amino groups in the alkali-treated collagen, and the increase in acid-binding capacity (0.06 mmol./g.) must presumably be due to release of basic groups other than amino groups. It is possible that these are imino groups, derived from hydrolysis of peptide links involving proline or hydroxyproline. Whilst this would suggest that such peptide links are more alkali-labile than those involving amino groups, Gordon, Martin & Synge (1943), from a study of the partial acid hydrolysate of gelatin, inferred that they were resistant to acid hydrolysis at 37°.

Although there is only a small increase in the number of terminal groups in the alkali-treated collagen, an appreciable amount (5 %) of the collagen was rendered soluble by the treatment. Some of this was presumably in the form of large polypeptides, since it was precipitated on neutralization of the solution; peptides which did not precipitate probably account for nitrogen in the solution in excess of that due to ammonia and urea. The amino-N figure for the solution suggests that this fraction must be in the form of amino-acids or small peptides. Evidence deduced from the titration curve suggests that this soluble fraction is rich in

the dicarboxylic acids. Martin (1946) has recently demonstrated the presence of aspartylglutamic, glutamylglutamic, and glutamylaspartic acids in the partial hydrolysate of wool, and if these two dicarboxylic acids also occur together in collagen, breakdown could readily lead to their specific loss.

Consideration of the swelling curves indicates that physical as well as chemical changes take place during the alkaline treatment, for the changes in the curve can only be partially accounted for by changes in the reactive groups. The water uptake in the isoelectric range is increased, the increase in water uptake in acid solutions is greater than corresponds to the increase in basic groups, and the increase between pH 7 and 9 can only partially be accounted for by the titration of uncharged carboxyl groups liberated by hydrolysis of amide groups. The cause of the shift in the pH at which water uptake in alkaline solutions reaches a maximum from 12.5 to 9.5 is not clear.

Examination of the commercially treated skins indicates that similar changes have taken place; the total N and amide N of these were lower than those of the original ox-hide collagen, but the amino N was in some cases lower, instead of higher as might have been expected from the laboratory experiment. It is possible that this may be due to bacterial deamination at some stage in the processing of the skins (Stuart, 1946). The total N of the sheep skins was particularly low, and is not accounted for by the lower values of the amide N and arginine N as compared with the original ox-hide collagen, which suggests the possibility that the composition of sheep-skin collagen may differ from that of ox hides.

SUMMARY

1. Hydrolysis of amide groups is the main reaction taking place when collagen is treated with alkali at pH 13.0. A small number of the guanidino groups is converted to ornithine and urea, and a still smaller fraction to citrulline and ammonia. There is a small increase in the total number of basic groups and in the number of free amino groups; it is suggested that some peptide links involving proline and hydroxyproline are broken. The hydroxyamino-acids, serine, threonine and hydroxylysine, are unaffected by the treatments. Approximately 5 % of the collagen is solubilized.

2. Alkali-treated collagen swells more than the original collagen.

3. Skins treated commercially with alkaline solutions undergo changes similar to those found in laboratory experiments.

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The Nutrition of the Larva of *Aedes aegypti* Linnaeus

3. LIPID REQUIREMENTS

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In a previous communication (Golberg, De Meillon & Lavoipierre, 1945) we showed that water-extracted yeast residue, supplemented by a number of water-soluble vitamins, constitutes an adequate medium for the growth and metamorphosis of the larva of *Aedes aegypti* Linnaeus. From this starting point our investigations were concerned with the part played by lipids in the nutrition of the mosquito larva.

Considering the ability of insects to transform carbohydrate and protein into fat (evidence summarized by Wigglesworth, 1942), it is surprising to find that many insects are incapable of synthesizing sterols; or at least of producing them in sufficient quantities to cover their needs for growth and particularly for metamorphosis. The need for pre-formed sterol in the diet of many insects has been conclusively demonstrated. Yet serious consideration does not appear to have been given to the possibility that non-steroid lipids may be capable of fulfilling the same nutritive purpose. For example, iecithin has been employed as a component of diets for *Drosophila* (Bacot & Harden, 1922; Guyénot, 1917; Lafon, 1937), and frequent reference is made in the literature to essential lipid factors present in wheat, wheat germ, cod-liver oil, butter fat, and ethanolic extract of yeast. In two instances, in which

attempts have been made to fractionate the fat and isolate the active agent, the conclusion was reached that cholesterol was the essential constituent (Hobson, 1935; van't Hoog, 1936). Fraenkel & Blewett (1945) found that, in addition to cholesterol, insects of the genus *Ephestia* require linoleic acid and vitamin E.

Gay (1938) and subsequent workers (Fraenkel, Reid & Blewett, 1941; Fraenkel & Blewett, 1943) employed yeast in the growth media used for studying the sterol requirements of insects. Although the proportion of yeast in the dietary solids varied from 5 up to 96%, they, nevertheless, found it necessary to add a further quantity of cholesterol to the diet, the minimum being 1 mg. sterol/3 g. diet (Fraenkel *et al.* 1941). Dried yeast has a fat content of 7% (Newman & Anderson, 1933) of which approximately one third consists of sterols (Smedley-MacLean & Thomas, 1920). It would appear that this level of sterols is inadequate for insects normally feeding on flour or other stored food, particularly when the yeast constitutes only 5% of the artificial diet. In the case of *Aedes* larvae, however, when the whole of the solid portion of the growth medium was composed of yeast, no added sterols or other lipids were needed.