CXLIV. STUDIES ON COLLAGEN. THE CHANGES WHICH COLLAGEN UNDERGOES WHEN TREATED WITH SOLUTIONS OF HYDROCHLORIC ACID AND SODIUM HYDROXIDE[1].

By JOHN KNAGGS.

From the Biochemical Department, Imperial College of Science and Technology, S. Kensington.

(*Received September 24th, 1929.*)

It is a well-known fact that, if a protein is treated with an acid or an alkali, the physical and chemical properties are changed, the degree and nature of these changes depending on the acid or alkali used and on the hydrogen ion concentration.

The first workers in the field, Hardy [1905], Pauli [1922], and Laqueur and Sackur [1902], who investigated the globulins, albumins, and caseinogen respectively, considered that proteins with either an acid or an alkali formed ionisable salts, and attributed the observed increases in certain physical constants to the greater association between the ionised salt and water.

A considerable amount of work has been carried out on other proteins, especially gelatin, by numerous investigators, and from their results it may be concluded that at p_H 2–3, p_H 11–12, and at the isoelectric point there is either a maximum or minimum value for the physical constants, viz., osmotic pressure, viscosity, swelling, specific rotation, etc., and the theoretical aspect of the question has been discussed by Robertson [1920], Dakin [1910, 1912], Leuchs and Manasse [1907], Leuchs and La Forge [1908] and more recently by Levene, Bass, Steiger and Bencowitz [1927].

This paper is mainly concerned with the changes which occur in the precursors of gelatin, when they are treated with solutions of acid or alkali. The investigation is made more difficult by the fact that collagen is insoluble at ordinary temperatures and consequently cannot be brought into solution without altering its internal structure. Therefore most of the facts which are known relating to collagen have been obtained by a study of the gelatin extracted from it. The only simple physical property which can be determined directly is the swelling with change of hydrogen ion concentration. This has been carried out on hide powder by Porter [1921, 1922], Atkin [1922], Kubelka [1918], Kubelka and Taussig [1926], on goat-skin by Kaye and Jordan-Lloyd [1924, 1, 2], and on fish-skin by Kernot and Knaggs [1929].

[1] Thesis presented at the University of London for the Degree of Ph.D.

Knaggs and Schryver [1924, 2] showed that a gelatin derived from a precursor treated with alkali had on hydrolysis a higher percentage of basic nitrogen than one from an acid-treated precursor.

Further, Knaggs [1923] and Kernot and Knaggs [1928, 2] state that proteins treated with cold acid before hydrolysis give results for the Hausmann numbers which indicate that internal structural changes have taken place in the molecule of the protein which markedly affect the distribution of nitrogen, especially in the diamino-fraction.

The author concluded from the above results that, when a tissue swells in solutions of acid or alkali, the swelling is accompanied by intramolecular changes in the molecule of the protein, especially in acid below p_H 2·2 and in alkali above p_H 10·5.

From the work of Jordan-Lloyd and Mayes [1922] and Atkin and Douglas [1924] on the combining power of gelatin with HCl or NaOH, it is noticed that at hydrogen ion concentrations of greater than p_H 1·7 and less than p_H 10 there is a considerable increase in the amount of acid or base fixed.

It was therefore decided to study the precursors of gelatin in order to throw some light on the physical and chemical changes which take place when they are treated with solutions of HCl and NaOH above 0·03 %, and, in particular, 0·5 % HCl and 0·2 % NaOH as these are the concentrations of acid or alkali used industrially, when purifying animal tissues previous to the extraction of the gelatin.

With this object in view the investigations were carried out on the following lines and, to simplify the problem, the collagen used in all these experiments was purified as far as possible from all inorganic and tissue impurities using the method of Knaggs and Schryver [1924, 1].

(1) The purified collagen was treated with 0·5 % HCl or 0·5 % NaOH for a known length of time. The acid or alkali was then removed and the gelatin extracted.

The Hausmann numbers of the gelatin and its precursor, and certain physical properties of the gelatin, were determined to show how far the treatment of the precursor influenced the physical properties and chemical constitution of the gelatin.

(2) The alkali-treated precursor was digested with trypsin to remove the elastic fibres in order to obtain a comparatively pure collagen, and the Hausmann numbers of the collagen so obtained and the gelatin extracted from it were determined.

(3) The swelling of

(*a*) an acid-treated precursor,

(*b*) an alkali-treated precursor,

(*c*) a precursor free from elastic fibres,

in different concentrations of HCl and NaOH was studied and the Hausmann numbers of the swollen tissue were determined, especially in the region of maximum swelling.

Experimental.

Preparation of the materials.

The femur, Achilles tendon and horn-pith of a freshly killed ox were the starting materials. The femur and horn-pith were decalcified by 3 % HCl, and the acid was removed by washing in running water. They then received treatment with 0·2 % NaOH for a prolonged period to remove, as far as possible, all organic impurities [Knaggs and Schryver, 1924, 1].

In the ossein and horn-pith there still remained traces of calcium phosphate which was removed by Smith's method [1921], which consists in treating the tissue with saturated brine and 0·5 % HCl. After washing it, the last traces of electrolyte were removed by electrolysis for 10 days, using the apparatus described by Knaggs, Manning and Schryver [1923] for the purification of gelatin. The collagen thus obtained was practically free from ash.

Method of analysis used.

This consisted in the determination of the Hausmann numbers by the method of Osborne and Harris [1903]; the exact procedure is given in the paper by Knaggs and Schryver [1924, 2].

Unless otherwise stated the hydrolysis of the protein was carried out by 20 % HCl for 20 hours, taking the precaution to hydrolyse immediately with boiling acid [Knaggs, 1923], and to precipitate the basic nitrogen by phosphotungstic acid under exactly the same conditions of temperature, concentration of nitrogen in solution, etc., to eliminate as far as possible those errors which are likely to occur and are fully described by Kernot and Knaggs [1928, 2].

The effect of HCl and NaOH on the precursors of gelatin.

The precursors were treated for 5 days with 0·2 % NaOH or 0·5 % HCl, and the alkali or acid was removed by washing in running water. A part was electrolysed to remove all electrolytes and the gelatin was extracted with water from the remainder, and was then purified by flocculating twice by means of an electric current [Knaggs and Schryver, 1924, 1]. The analytical results are given in Table I.

Table I.

Horn-pith	Amide-N	Humin-N	Diamino-N	Mono-amino-N	Non-amino-N
A. Treated with 0·2 % NaOH for 5 days, well washed and dried	2·08	0·95	24·49	56·60	15·88
A was electrolysed for 10 days	1·97	1·12	22·88	56·24	17·79
Gelatin from *A* twice flocculated	1·30	0·66	24·20	58·64	15·20
B. *A* treated for 5 days with 0·5 % HCl, washed and dried	1·68	0·90	22·45	58·85	16·12
B was electrolysed for 10 days	1·47	1·73	20·71	58·23	17·86
Gelatin from *B* flocculated twice	1·45	1·82	22·94	58·22	15·57
Tendon					
C. Tendon treated with 0·2 % NaOH and electrolysed 10 days	3·51	0·83	25·13	55·18	15·35
Gelatin from *C*	3·02	0·79	29·50	52·12	14·57
D. Tendon treated with 0·5 % HCl and electrolysed 10 days	4·18	0·69	22·30	56·80	16·03
Gelatin from *D*	3·45	0·99	26·00	53·57	15·99

The Hausmann numbers for gelatin extracted from precursors of mammalian origin have already been given in the paper by Knaggs and Schryver [1924, 2]. A conclusion similar to theirs can be drawn regarding the precursor from the figures in Table I, viz. that a precursor treated with alkali gives on hydrolysis a higher percentage of diamino-nitrogen than one treated with acid.

Further, a precursor undergoes some internal structural change under the influence of an electric current: for example, horn-pith treated with either acid or alkali shows a decrease of approximately 2 % in the percentage of diamino-nitrogen, while a slight decrease in the percentage of amide-nitrogen after electrolysis is noticed. The distribution of nitrogen in gelatin from horn-pith differs very slightly from that in its precursor, except that, in the former, the percentage of amide-nitrogen is less, due no doubt to the removal of amino-groups during extraction. On the other hand, tendon gelatin and its precursor show a considerable difference in the percentage of diamino-nitrogen.

Precursors treated with trypsin.

The precursor used above contained elastic fibres (elastin) whose properties and chemical composition are very different from those of gelatin. Elastin is insoluble in most ordinary reagents, and Richards and Gies [1902] showed that on hydrolysis only 3·34 % of the total nitrogen was present as basic nitrogen, and Kossel and Kutscher [1898] obtained only a minute quantity of arginine from it.

The elastin was therefore removed from the tissue, before extracting the gelatin, by digestion with trypsin. Wilson [1920], Marriott [1921] and Rohm and Haas Co [1922, 1923] have shown that when a mammalian skin is digested with trypsin the elastic fibres only are attacked and collagen remains.

Taking advantage of this fact, ossein, ox-tendon, horn-pith, shark-skin and tendon from the head of a sperm-whale, which had been treated for a long period with 0·2 % NaOH, were digested for 20 days with trypsin in sodium carbonate solution of p_H 8. Alkali-treated precursors only were used as it has been shown by Kühne and Ewald [1877] and Reich-Herzberge [1901] that trypsin will attack acid-treated collagen [see also Plimmer, 1912, and Northrop, 1921, 1, 2].

The tissues were well washed in running water and the gelatin was extracted. By this method it was assumed that practically pure collagen was obtained free from ash and elastic fibres. The precursor and the gelatin extracted therefrom were purified as above and the Hausmann numbers of each determined (Table II).

As well as digesting the elastic fibres, the action of trypsin on the precursor produces changes in the collagen molecule. This is shown to be the case by comparing the results given in Tables I and II for horn-pith. Alkali-treated horn-pith has a higher percentage of diamino-nitrogen than that treated with trypsin, the change being from 22·88 to 20·81. The gelatins obtained from each show marked differences, especially in the basic fraction,

Table II. *Hausmann numbers of gelatin and its precursors, the precursors having been treated with trypsin.*

	Amide-N	Humin-N	Diamino-N	Monoamino-N	Nonamino-N
A. Ossein	2·04	2·09	23·66	57·85	14·36
A_1. Gelatin from *A*	2·10	0·85	25·77	59·03	12·25
B. Horn-pith	2·10	5·13	20·81	55·19	16·87
B_1. Gelatin from *B*	1·79	0·98	26·79	58·25	12·19
C. Shark-skin	3·92	1·68	24·60	57·92	11·88
C_1. Gelatin from *C*	3·43	1·41	31·53	46·45	17·18
D. Whale-tendon	3·74	3·88	22·60	64·34	5·44
D_1. Gelatin from *D*	2·88	1·25	24·77	60·85	10·25

which is very much greater when the precursor has been treated with trypsin. In general the action of trypsin on collagen is to increase the percentage of humin-nitrogen at the expense of the diamino-fraction. The Hausmann numbers of the gelatins extracted from enzyme-treated collagens show a normal value for the humin-nitrogen, but the percentage of basic nitrogen is very high. Sadikoff [1903, 1904] showed that gelatins from tendon, previously treated with trypsin and then with various strengths of alkali, differed from each other in physical properties. It is quite possible that these variations were due to the p_H of the solutions of alkali used.

THE VARIATIONS IN THE PHYSICAL PROPERTIES OF GELATINS EXTRACTED FROM A PRECURSOR TREATED WITH HCl OR NaOH SOLUTIONS OF DIFFERENT CONCENTRATIONS.

The following experiment was carried out with the object of trying to find in what way the intramolecular changes in the collagen molecule influenced the physical properties of the gelatin obtained from it.

50 g. of purified horn-pith were treated with 2 litres of 2 % NaOH solution for 5 days. The horn-pith was then thoroughly washed to remove all the alkali, and the gelatin, extracted at 80°, was purified by flocculation as before.

The process was repeated with other samples of horn-pith using 1, 0·5, 0·2 % NaOH solutions, and HCl solutions of the same concentration as those of the alkali. A 5 % solution of each of these samples of gelatin was prepared, and the viscosity at 40° was determined. The drop-number for soya bean oil was also determined by the methods used by Kernot and Knaggs [1928, 2].

Estimations of amino-nitrogen before hydrolysis and of nitrogen precipitated by phosphotungstic acid after hydrolysis were carried out for each sample. The results are given in Table III and shown graphically in Fig. 1.

The curves *A*, *B* and *C* (Fig. 1) for drop-number, percentage of free amino-groups, and of nitrogen precipitated by phosphotungstic acid from the hydrolysed gelatin respectively are roughly of the same shape and follow one another fairly closely. For example, there are maxima around the region of 0·2 % and 1 % on the acid side and 1 % on the alkali side. The minima occur at 1 % acid and 0·5 % alkali. The viscosity curve *D* may roughly be described as the mirror image of *A*, *B* or *C*. In each case the minimum values indicated on the viscosity curve are accompanied by corresponding maximum values on the other curves.

Table III.

Treatment of horn-pith before extraction	p_H	5 % solution: Drop-number for soya bean oil	5 % solution: Viscosity	Gelatin not hydrolysed. Amino-N %	Hydrolysed by 20 % HCl. Diamino-N %*
2·0 % HCl	0·35	64	2·634	4·96	35·43
1·0 % „	0·72	59	3·449	4·78	29·4
0·5 % „	1·10	61	2·944	4·86	32·3
0·2 % „	1·66	64	2·702	4·50	32·2
0·2 % NaOH	12·57	62	6·040	4·31	28·3
0·5 % „	13·3	64	4·385	4·38	26·8
1·0 % „	13·56	68	2·897	5·27	28·7
2·0 % „	13·67	66	4·033	4·78	35·14

* In this case the amide- and humin-nitrogen were not removed before precipitating the basic fraction. The HCl was removed by distillation under reduced pressure, and the residue dissolved in water. The precipitation with phosphotungstic acid was then carried out under the same conditions as described above.

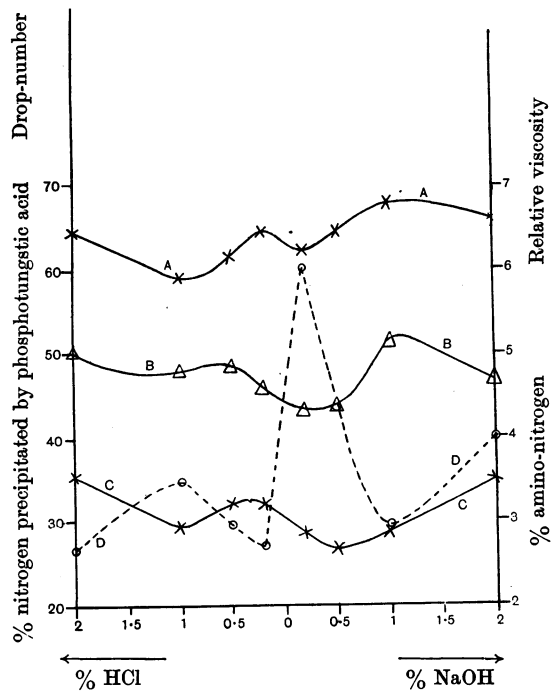


Fig. 1. Final treatment of precursor before extraction of gelatin. Purified gelatin from horn-pith.

A. Drop-number, ×. *B*. % amino-N, △.

C. % N precipitated by phosphotungstic acid after hydrolysis with 20 % HCl, ×.

D. Relative viscosity, ⊙.

It was pointed out by Kernot and Knaggs [1928, 2] that the emulsifying power of a sample of gelatin depended on the previous treatment of the precursor, a gelatin from an alkali-treated precursor being the more efficient, and they concluded that this was due to the higher viscosity and to the presence of more free amino-groups in the gelatin molecule. From the curve in Fig. 1 this conclusion may be extended to include the percentage of basic

nitrogen, which also varies with the drop-number. This is in agreement with the conclusion of Van Slyke and Birchard [1913] who state that the percentage of amino-nitrogen is equal to half that of the lysine-nitrogen in the protein.

It is interesting to note that the changes in the viscosity of gelatin are greatest when the precursor is first treated with acid or alkali and purified before extraction. Schroeder [1903] obtained results of a lower order, owing to the fact that the acid or alkali was not removed from the treated gelatin before the determinations were carried out.

These increases in the viscosity of gelatin due to the effect of acid and alkali have been explained by assuming that the molecules of the protein either form aggregates or become more highly hydrated in these solutions. The enormous increase in the viscosity of gelatin from a precursor treated with alkali, particularly 0·2 % NaOH, cannot be explained solely in this way. It is more likely to be due to the collagen molecule undergoing intramolecular re-arrangement, the nature and degree of such changes having a direct relationship to the strength of the acid or alkali used to treat the precursor.

The relationship between the swelling of a precursor in solutions of NaOH and HCl, and the changes in the chemical constitution which accompany the swelling.

This section is devoted to a study of the changes which occur in collagen when it is treated with solutions of NaOH or HCl. The results in Tables III and IV show that if the NaOH or HCl is not removed before hydrolysis, similar changes to those already mentioned in the Hausmann numbers occur, *i.e.* there is an increase in the percentage of basic nitrogen with both acid and alkali treatment, greater with the latter.

If the precursor is treated as above and hydrolysed with 5 % HCl instead of 20 % HCl other values for the Hausmann numbers are obtained as shown by the results in Tables IV and V for horn-pith and whale-tendon.

Table IV.

Horn-pith	Amide-N	Humin-N	Diamino-N	Mono-amino-N	Non-amino-N
A. Treated with 0·2 % NaOH, washed and dried	2·08	0·95	24·49	56·04	16·44
B. *A* electrolysed	1·97	1·12	22·88	56·24	17·79
B treated with 0·5 % HCl and hydrolysed without washing	1·63	0·38	23·64	59·02	15·33
B treated with 0·5 % NaOH and hydrolysed without washing	1·36	1·51	26·74	52·52	17·87
C. *A* treated with 0·5 % HCl, washed and dried	1·68	0·90	22·45	58·85	16·12
D. *C* electrolysed	1·47	2·73	20·71	57·23	17·86
D treated with 0·5 % HCl and hydrolysed without washing	1·51	0·87	24·07	55·34	18·21
D treated with 0·5 % NaOH and hydrolysed without washing	1·41	1·44	26·16	53·37	17·62

Whale-tendon. The whale-tendon was well washed and cut up into small pieces. It was treated for 10 days with 0·2 % NaOH, the solution being changed daily. It was only necessary to treat the material for a short time with alkali as the amount of chondroitinsulphuric acid present was very much smaller than in ox-tendon. The tendon was well washed, dried, and treated as described below, the Hausmann numbers being determined after each treatment.

Table V.

	Hydrolysed by	Amide-N	Humin-N	Diamino-N	Mono-amino-N	Non-amino-N
E. Dry whale-tendon	20 % HCl	4·23	0·98	25·84	66·12	2·83
E treated 5 days with 0·5 % HCl	20 % „	4·82	2·21	23·86	65·21	3·90
	5 % „	3·84	3·95	27·68	63·59	0·94
E treated 5 days with 0·5 % NaOH	20 % „	3·17	2·11	30·36	58·32	6·04
	5 % „	4·73	4·01	29·82	50·54	10·90

Knaggs [1923] and Kernot and Knaggs [1928, 2] showed that treatment of a protein with cold 20 % or 5 % HCl before hydrolysis caused structural changes in gelatin or its precursors. The above results indicate that 0·5 % HCl and 0·5 % NaOH produce other changes, from which it may be concluded that the nature and degree of the change depend on the strength of the acid or alkali used. This fact was utilised to try to elucidate the changes which occur in tendon and ossein during swelling with different concentrations of HCl or NaOH. The following experiments were therefore carried out, using tendon and ossein.

Swelling of tendon and ossein.

About 1 g. of the precursor was weighed out in a Gooch crucible. The crucible was suspended in a solution of HCl or NaOH of known p_H, contained in a wide-mouthed bottle of approximately 400 cc. capacity. The bottles were kept submerged to the neck in a thermostat at 25° for 48 hours, after which the crucibles were removed and the collagen was rapidly dried with filter paper and weighed. The p_H of the liquid was taken and the amount of nitrogen which diffused from the collagen into the solution was determined in each case by Kjeldahl's method.

For tendon the range of p_H was between 0 and 14, but the swelling of ossein was carried out using solutions of HCl and NaOH above N concentration, *i.e.* up to 20 %, because this precursor was treated with similar concentrations of cold acid and alkali before hydrolysis.

The high concentrations of acid or alkali are given as percentages, and the original concentration is used in plotting the curves. The results for swelling and the diffusion of nitrogen are shown graphically in Fig. 2, and these are corrected to 14 % of moisture for each tissue used.

The curves for the change in the swelling of tendon and ossein with p_H are very similar to those reported for other tissues and proteins by other investigators—Kaye and Jordan-Lloyd [1924, 1, 2] for goat-skin, Porter [1921, 1922] for hide-powder, Jordan-Lloyd [1920] for gelatin, Loeb [1920–21] for caseinogen, Fischer and Hooker [1918], Tolman and Stearn [1918] for

blood-fibrin and Jordan-Lloyd [1916] for the sterno-cutaneous muscle of a frog. Their work shows that the maximum swellings occur at an external p_H between 2–3 and 11–12 and the minimum swelling at the isoelectric point. The results for tendon and ossein are very similar to those above, viz. the maximum swelling for tendon on the acid side is at p_H 1·2 for the alkali-treated tissue and at p_H 1·5 for the acid-treated tissue, but no well-defined point for the minimum swelling is observed between p_H 4 and 10 as it varies very little. Tendon shows no maximum on the alkaline side. For ossein the maxima occur at p_H 0·6, 2·2 and 12·1, while the curve is very like that given for goat-skin treated with trypsin by Kaye and Jordan-Lloyd [1924, 2].

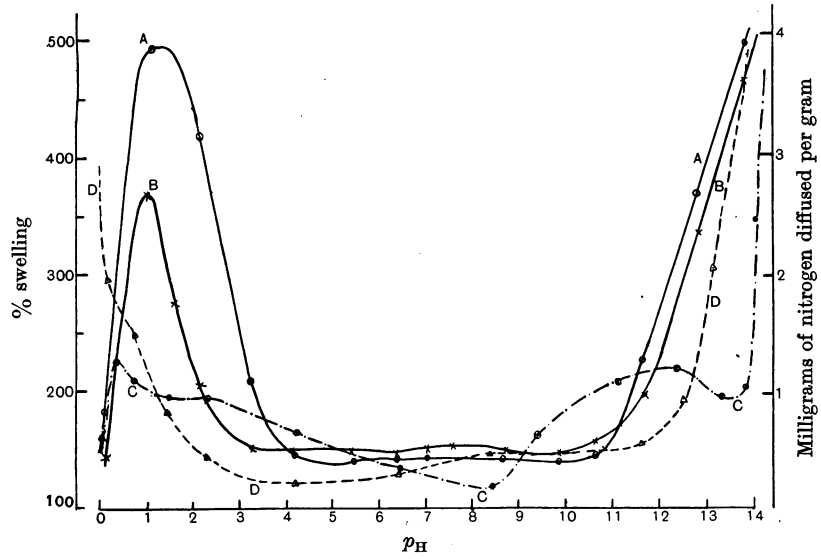


Fig. 2. Swelling of tendon and ossein in HCl and NaOH solutions.

———— *A*. Tendons treated with HCl, ⊙.
▬▬▬ *B*. Tendons treated with NaCl, ×.
—•—•— *C*. Ossein treated with trypsin, ⊕.
– – – – – – *D*. Milligrams of nitrogen diffused per gram of ossein, △.

The minimum value for ossein is at p_H 8·5, but there is no minimum shown on the acid side of the curve. The number of readings taken between p_H 4 and 10 does not warrant any definite conclusion being drawn from this fact, but it is interesting to note that Wilson and Kern [1923] claim that ash-free gelatin has a second isoelectric point at p_H 7·7. Thomas and Kelly [1925] hold the same view both for gelatin and collagen (hide-powder). Ossein treated with trypsin may have a different isoelectric point from collagen which has had no such treatment. It was shown by Gerngross and Bach [1923] that the isoelectric point of gelatin varied between p_H 4·45 and 5·55 according to its source. They also showed that treatment with formaldehyde lowered the isoelectric point of one sample of gelatin from p_H 5·05 to 4·6, and with another sample from 4·75 to 4·3. This point about ossein requires further investigation.

At p_H 0 to 4 and 10 to 14 acid-treated tendon swells more than the alkali-treated precursor; but this is reversed between p_H 4 and 10 (see Fig. 2). Bracewell [1919], from a study of the amount of acid absorbed by proteins (gelatin, fibrin, caseinogen, gliadin, edestin), concluded that this absorption is determined mainly by the content of lysine and arginine and is roughly proportional to the number of amino-groups per gram of protein.

No doubt this is the case for collagen at low concentrations of acid and alkali. By analogy with gelatin the alkali-treated tendon would swell more than that treated with acid, which is in agreement with the curves *A* and *B*, Fig. 2, but only between p_H 4 and 10. At higher concentrations of acid and alkali other factors come into play which considerably alter the degree of swelling of tendon.

Unfortunately there is no reliable method for the direct determination of the free amino-groups in collagen on account of its insolubility.

Between p_H 1 and 13 the amount of nitrogen which goes into solution is very small and, from Fig. 3 for ossein, it will be noticed that the collagen begins to disperse only at high concentrations of acid or alkali.

The hydrolysis of ossein and tendon.

The behaviour of tendon and ossein near the points of maximum swelling is not properly understood, but there is no doubt that the protein undergoes internal structural changes, the first result of which is the swelling and the second the dispersion of the tissue into solution. The following experiments were carried out with tendon and ossein to try to elucidate the changes which occur.

Tendon. The acid- and alkali-treated tendons were kept for 4 days in the thermostat at 20° with solutions of NaOH and HCl of concentrations between 0·125 % and 1 % and a blank was carried out, using boiled distilled water.

The tendon was quickly removed from the liquid, thrown into boiling 20 % HCl, and the heating continued for 20 hours. The Hausmann numbers of the hydrolysate were determined, taking the precautions mentioned above. The basic nitrogen was always precipitated from 100 cc. of solution which contained approximately 180 mg. of nitrogen.

The results obtained are plotted in Fig. 3 and on the same curve, for comparison, are included the percentage swellings of tendon, taken from Fig. 2.

The treatment of tendon with cold solutions of NaOH or HCl, followed by immediate hydrolysis with hot 20 % HCl, alters the distribution of nitrogen in a similar way to that observed with horn-pith.

Diamino-nitrogen. The action of cold NaOH on tendon is to increase the basic nitrogen while that of cold HCl decreases this value. It will be noticed that curves *A* and *B* (Fig. 3) are very similar in shape.

Nonamino-nitrogen. As this value is obtained by difference, no satisfactory conclusions can be drawn from the numbers unless there are large variations.

This is the case with alkali-treated tendon, there being a gradual decrease with cold HCl, while with cold NaOH the percentage of nonamino-nitrogen decreases rapidly as the concentration of NaOH increases, due probably to the ring compounds being converted into open chain compounds by the action of the alkali. With the acid-treated precursor, the nonamino-nitrogen increases with either HCl or NaOH but the changes are not large.

These differences in behaviour must be due to the different molecular structure of the two precursors.

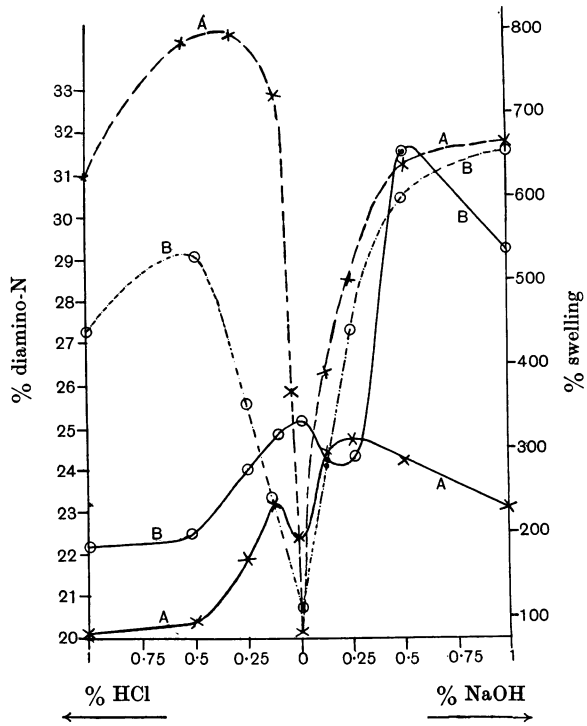


Fig. 3. Achilles tendon (ox). Air dried.

A. Final treatment with HCl solution, ×.

B. Final treatment with NaOH solution, ⊙.

——— Diamino-N after hydrolysis with 20 % HCl.

— — Swelling, *A*. —•—•— Swelling, *B*.

Swelling of the tendon.

The points of inflection on the curves *A* and *B* (Fig. 3) for swelling and percentage of diamino-nitrogen are given in Table VI.

The results obtained indicate that, when a tissue swells in acid, the internal structure of its molecule is altered, and, generally, as the concentration of the acid is increased the percentage of diamino-nitrogen decreases. At the point of maximum swelling the percentage of basic nitrogen is a minimum. With alkali the diamino-nitrogen increases, and it follows that, at the point of maximum swelling, the percentage of diamino-nitrogen is also a maximum.

Table VI.

Alkali-treated tendon				Acid-treated tendon			
Diamino N %		Swelling		Diamino-N %		Swelling	
Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.
0	0·5 % HCl	0·4 % HCl	0	0·125 % HCl	0·5 % HCl	0·5 % HCl	0
0·5 % NaOH	0·25 % NaOH	0·75 % NaOH	0	0·25 % NaOH	0	0·75 % NaOH	0

Hydrolysis of ossein free from elastin.

An experiment similar to the above was carried out, using ossein from which the elastin had been removed by trypsin. In this case the range of concentrations of the acid and alkali used was greater, from 20 % to 0·66 %.

10 g. of ossein were treated with 300 cc. of 0·66 % HCl for 4 days at 20°; the supernatant liquid and a part of the protein were heated for 20 hours at the boiling-point, and the remainder was hydrolysed with 20 % HCl (300 cc.). This was repeated, using the other concentrations of HCl and NaOH given in Fig. 3.

With 15 % and 20 % HCl and NaOH, a large part of the ossein dissolved, so that only the liquid was heated, and the undissolved part was hydrolysed by 20 % HCl.

Two sets of results were thus obtained (Fig. 4), one in which the ossein had been treated throughout the experiment with the same strength of acid or alkali, and another where the ossein, after treatment with cold acid or alkali, was hydrolysed by 20 % HCl.

Hydrolysis of ossein after treatment with cold HCl or NaOH.

(*a*) *With* 20 % *HCl.* The products of hydrolysis of ossein vary considerably according to the previous treatment, in a similar way to that observed with tendon and horn-pith. The amide-nitrogen varies slightly. An increase is noticed on the acid and a slight decrease on the alkaline side. With 20 % NaOH the percentage falls to 0·8 showing that only very concentrated solutions of alkali hydrolyse the free amino-groups in the cold.

The percentage of diamino-nitrogen is plotted against the concentration of acid or alkali used in treating the ossein before hydrolysis in Fig. 4. It is noticed that there is a minimum value for 10 % HCl, and the curve ascends rapidly as the strength of acid is increased or decreased, the maximum value being with 2 % HCl.

There is a second minimum at zero which corresponds to the ossein which had been treated with water only.

On the alkaline side there is one maximum at 2·5 % NaOH.

(*b*) *With the same strength of acid or alkali with which the ossein was treated in the cold.* Referring to the curves in Fig. 4, it is noticed that all three curves are similar in form; for example, considering the curves *A* and *B*, there

is a fairly constant difference between them, and it is only near the isoelectric point that this difference diminishes.

The zero point for curve *A* is not theoretically correct and is obtained by continuing the two branches of curve *A*. Theoretically this should be 100, on the assumption that when pure collagen is treated for 4 days with pure water and then heated for 20 hours no hydrolysis occurs and the whole of the protein is therefore precipitated by phosphotungstic acid.

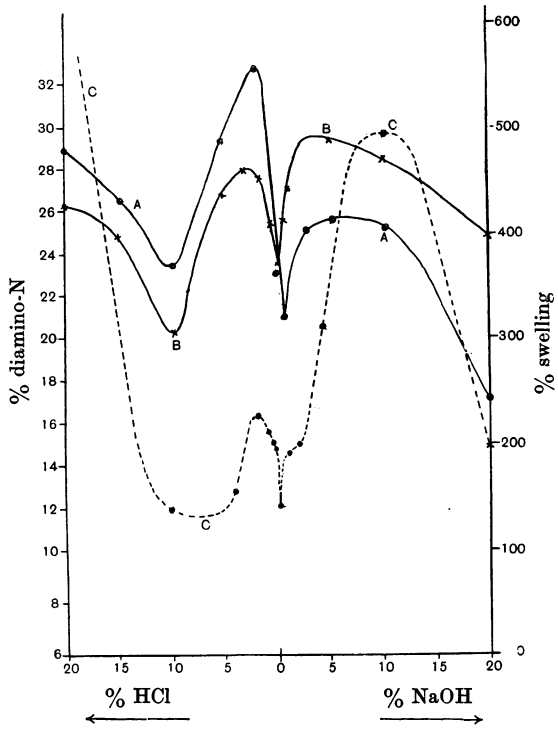


Fig. 4. Ossein (free from elastin).

A. Percentage of diamino-N, after standing in contact with HCl and NaOH of different strengths, indicated on abscissae, and hydrolysed with acid or alkali of the same concentration.

B. Percentage of diamino-N, after standing as for *A*, but ossein hydrolysed with 20 % HCl.

C. Swelling in acid and alkali of different concentrations.

The results for the hydrolysis of ossein with different concentrations of HCl are somewhat similar to those obtained by using 20 % HCl, the chief differences being in the higher percentage of basic nitrogen, and in the percentage of nonamino-nitrogen decreasing as the concentration of acid is increased.

The hydrolysis with NaOH is very different, the final products no doubt being different. The percentage of diamino-nitrogen is less than that obtained when 20 % HCl is used for hydrolysis. There is a loss of nitrogen as ammonia, due to the scission of some of the amino-groups, especially at the higher

concentrations of NaOH, and during hydrolysis an insoluble nitrogen compound is formed. This was removed by filtration before the Hausmann numbers were determined.

Fosse [1912] showed that when a protein was hydrolysed by alkalis, the arginine was split into urea and ornithine. The former would be decomposed by the alkali into ammonia, while the latter would be precipitated by phosphotungstic acid. Ornithine contains a lower percentage of nitrogen than arginine, and in consequence the percentage of basic nitrogen would be lower, which is in agreement with the results (Table X).

As stated above the effect of cold NaOH appears to cause scission of the rings of closed chain compounds. This effect is more marked with hot alkali, for it is noticed that the percentage of nonamino-nitrogen decreases gradually as the concentration of the NaOH is increased, until with 20 % NaOH it is reduced to zero.

The relationship between the degree of swelling and the products of hydrolysis of tendon and ossein.

On comparing the curves in Figs. 3 and 4 for the percentage of diamino-nitrogen of ossein and tendon after hydrolysis, using 20 % HCl, it is noticed that they resemble one another in shape although the range of concentration of acid and alkali used for tendon is not so great. Tendon is much more sensitive to a small change in p_H than ossein, especially on the acid side. A striking point about the swelling curve for ossein is that, at the point of maximum swelling, the percentage of diamino-nitrogen is a maximum on the acid side (with tendon this is a minimum), but, on the alkaline side, the highest value for the basic nitrogen is at the point on the curve where the swelling begins to increase very rapidly. The percentage of diamino-nitrogen then decreases gradually. At the maximum swelling there is a tendency for some of the protein to become soluble, and it may be that these changes in the distribution of nitrogen in the collagen molecule result in making the protein soluble in HCl or NaOH solutions.

Hydrolysis of fibrin from ox-blood.

An experiment similar to that just described for ossein was carried out, using fibrin from ox-blood. The fibrin was prepared as follows [see Hammarsten and Hedin, 1924]. The freshly drawn blood was thoroughly beaten and the elastic fibrous masses were well washed in running water. It was obtained free from blood corpuscles, etc. by washing several times with 5 % sodium chloride solution and finally with water. The fibrin was dried by blotting paper, and the following results were obtained (Fig. 5). The solution from which the diamino-nitrogen was precipitated by phosphotungstic acid contained 75 mg. of nitrogen (approximately).

No results are given for the swelling of fibrin, as this substance easily

disintegrates in strong solutions of HCl, and readily dissolves in a solution of NaOH.

The results for the percentage of diamino-nitrogen are shown in Fig. 5, and the curves resemble those for tendon and ossein (Figs. 3 and 4).

It will be noticed that in the curve *B* for hydrolysis, using 20 % HCl, there are maxima at 2·5 % HCl and 1 % NaOH and minima at 10 % HCl, 0·5 % and 3·3 % NaOH, and for curve *A* similar points of inflection occur at the same concentrations except on the acid side, when the maximum is at 0·66 % HCl, being no doubt due to incomplete hydrolysis.

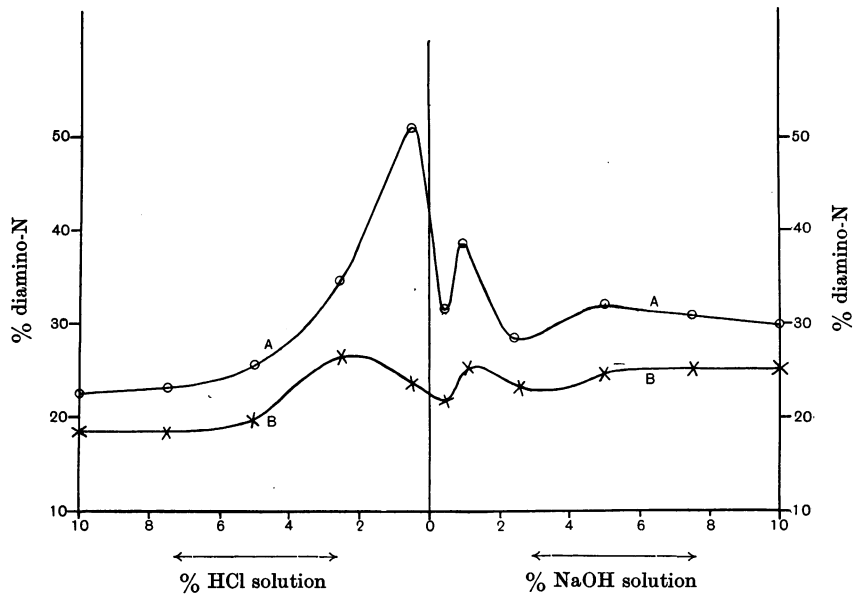


Fig. 5. Fibrin from ox-blood.

A. In contact with a solution of HCl or NaOH of varying strength for 4 days at 20° and hydrolysed at 100° with same concentration of acid or alkali, ⊙.

B. Same as *A* but hydrolysed at 100° with 20 % HCl, ×.

Curves *A* and *B* (Fig. 5) do not cross, as they do in the case of ossein (Fig. 4). Curve *A* is always above *B* even when NaOH is used as the hydrolytic agent. This may be due to the relative amounts of lysine, arginine and histidine being different in ossein and fibrin. The analytical results obtained by several investigators are given in Tables VII and VIII.

Table VII.

	Arginine	Histidine	Lysine	Observer
Fibrin	10·91	4·36	12·05	Gortner [1916]
Fibrin	13·14	3·96	11·4	Van Slyke [1911]
Gelatin	14·0	3·59	6·20	Van Slyke [1911]

The results given in Table VII were obtained, using the method of Van Slyke [1911] for the estimation of the distribution of nitrogen in a protein.

Fibrin contains a lower percentage of arginine and a much higher percentage of lysine than gelatin, and also in this case the total diamino-nitrogen is greater.

Other workers obtained the following results by isolating the individual amino-acids after hydrolysis of the protein.

Table VIII.

	Arginine %	Histidine %	Lysine %	Observer
Fibrin	4·61	2·05	5·18	Lock and Thomas [1913]
,,	3		4	Kutscher [1898]
Gelatin	7·62	0·40	2·75	Hart [1901]
,,	8·2	0·9	5·9	Dakin [1920]

It is seen that the results for both gelatin and fibrin do not always agree. This may be due to the previous treatment these proteins received before hydrolysis, which probably altered appreciably their chemical constitution. It may be concluded from these results (Tables VII and VIII) that fibrin contains a much lower percentage of arginine than does gelatin, and that the decrease in the basic fraction when gelatin is hydrolysed by sodium hydroxide is due to the decomposition of the arginine into urea and orinthine.

Fibrin contains a much higher amount of amide-nitrogen than does gelatin, and consequently any changes which occur are more readily detected with this protein. It will be noticed that an increase in the concentration of NaOH causes the percentage of amide-nitrogen to diminish, and, whatever the concentration of alkali used, this value is always much less than when acid is used for the hydrolysis.

The low numbers obtained, using 1 % or 0·5 % HCl, are due to incomplete hydrolysis of the protein.

When fibrin previously treated with cold alkali is hydrolysed by 20 % HCl, it has a much higher percentage of amide-nitrogen than in the other cases, in this respect resembling collagen.

Discussion.

From the foregoing results it may be concluded that collagen, when treated with cold solutions of HCl or NaOH of different concentrations, undergoes internal structural changes especially at p_H 2 or less and p_H 11 or more, *i.e.* at and above the points of maximum swelling. Between p_H 3 and 10 the chief reaction which takes place is that the acid or alkali combines with the free amino- and carboxyl-groups, forming salts. At other concentrations free acid or alkali is present which probably brings about intramolecular change.

It is a well-known fact that, if the chemical constitution of a compound is altered, such changes are accompanied by variations in the physical properties of the substance. This may be attributed in the case of proteins primarily to salt formation and secondly to spatial re-arrangement of the atoms composing the protein molecule caused by the action of the acid or alkali.

These tautomeric changes in collagen take place slowly and the compounds formed are fairly stable. Dakin [1912] showed this to be the case from a study of the changes with time in the optical rotation of gelatin in alkaline solution.

The Hausmann numbers indicate that the action of cold HCl and NaOH on collagen is to produce fairly stable compounds whose molecules, on hydrolysis with boiling 20 % HCl, undergo scission at different points in the chain to yield different end-products. This is possibly due to the peptide linkages existing in two tautomeric forms, which break down in different ways on hydrolysis.

The evidence in favour of two or more isomers of collagen is as follows.

(1) The Hausmann numbers of acid-treated collagen differ in the percentages of amide-nitrogen and diamino-nitrogen from those of an alkali-treated collagen. The gelatins extracted from these precursors also differ in a similar way.

(2) The percentage swelling of collagen in HCl and NaOH of varying p_H values differs according to the previous treatment.

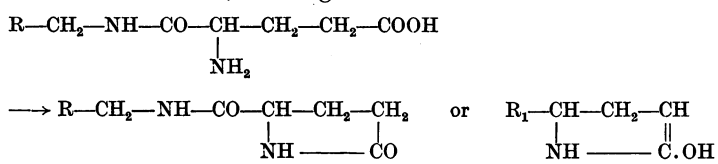
(3) The rate of extraction of gelatin from an acid-treated precursor is greater than from the same precursor which has had alkali treatment [Knaggs and Schryver, 1924, 2].

(4) Gelatin from an alkali-treated precursor has a higher percentage of free amino-groups than gelatin from an acid-treated precursor. The former has a higher viscosity and emulsifies animal or vegetable oils more easily than the latter [Kernot and Knaggs, 1928, 1].

(5) Treatment of acid- or alkali-treated collagen with cold solutions of HCl or NaOH of different concentrations and subsequent hydrolysis with 20 % HCl gives different hydrolysis products. The greatest variation is noticed in the percentage of basic nitrogen. A gelatin from alkali-treated collagen has a higher percentage of free amino-groups than one from a precursor treated with acid.

An explanation of the above is very difficult owing to the complex structure of a protein molecule, but several useful facts may be deduced.

On repeated flocculation of gelatin in an electric field or on heating the solution of ash-free gelatin, an increase in the percentage of nonamino-nitrogen is shown [Knaggs and Schryver, 1924, 3]. This is probably due to the glutamic acid residue which [see Dakin, 1912] occupied a position at the end of a branched chain, forming a lactam.



Such a group could not be estimated by the Van Slyke method.

Collagen does not behave in this way, as the percentage of nonamino-nitrogen does not increase when it is electrolysed for several days. It may

be assumed from this that probably the branched chains of gelatin are formed into rings in collagen [Procter, 1917; Procter and Wilson, 1923] and these rings are ruptured when the gelatin is extracted by hot water. Treatment with cold solutions of acid or alkali probably has the same effect.

This will no doubt explain the difference in the degree of swelling of gelatin and collagen. Kaye and Jordan-Lloyd [1924, 2] state that the volumes in cc. occupied after swelling by 100 g. vacuum-dried material at 20°, at p_H 2·3 for powdered gelatin and hide-powder are 11,550 and 3700 respectively and 2700 and 2100 respectively at p_H 5·1.

By using hide-powder the restraining influence on swelling, due to the fibrous structure of the skin, is removed and therefore the above numbers are comparable.

At the point of maximum swelling gelatin swells considerably more than collagen; this may be explained by assuming that the collagen molecule is mainly composed of closed rings, whereas in gelatin some of these rings are open. The acid or alkali forms salts and the evidence favours the view that these salts are highly hydrated [Pauli, 1922; Hardy, 1905].

The experimental results above seem to indicate that at the point of maximum swelling the rings of additional closed chain compounds are ruptured, which gives further points of attachment for the acid or alkali, and consequently an enormous increase in the hydration of the protein molecule. This is shown by the greater amount of the acid absorbed by gelatin [Jordan-Lloyd and Mayes, 1922], and also by the fact that, if a protein is treated with concentrated solutions of acid or alkali, it cannot be recovered again in the original condition.

Summary.

1. Samples of collagen and the gelatin extracted therefrom, derived from different mammalian tissues, were prepared in a highly purified state.

2. The collagens were treated with HCl and NaOH of varying concentrations for known periods. It was found that an alkali-treated precursor gave on hydrolysis a higher percentage of diamino-nitrogen than one treated with acid.

3. Ash-free gelatins were prepared from horn-piths differently treated. Curves are given showing how the previous treatment of the precursor affects the viscosity, drop-number and percentages of amino- and diamino-nitrogen. The viscosity curve is roughly the mirror image of the other three curves.

4. The elastin was removed from collagen by tryptic digestion, and the resulting product on analysis gave results which seem to indicate that the enzyme produces intramolecular changes in the protein molecule.

5. The swelling of collagen in HCl and NaOH of different p_H values was carried out. The hydrolysis of the swollen tissues was studied. The following conclusions were drawn from the results.

(*a*) The previous treatment affects the degree of swelling.

(*b*) Around the point of maximum swelling the percentage of diamino-nitrogen is either a maximum or a minimum. Therefore it is concluded that swelling is accompanied by internal structural changes and scission of closed rings. The latter gives rise to more free groups for the attachment of molecules of water.

(*c*) Collagen prepared from different tissues has not the same chemical constitution.

I wish to say how very grateful I am for the advice and help given me by the late Prof. Schryver during the course of this work.

REFERENCES.

Atkin (1922). *J. Ind. Eng. Chem.* **14**, 412.
—— and Douglas (1924). *J. Soc. Leather Trades Chem.* **8**, 359.
Bracewell (1919). *J. Amer. Chem. Soc.* **41**, 1511.
Dakin (1910). *Amer. Chem. J.* **44**, 48.
—— (1912). *J. Biol. Chem.* **13**, 357.
—— (1920). *J. Biol. Chem.* **44**, 499.
Fischer and Hooker (1918). *J. Amer. Chem. Soc.* **40**, 272, 292.
Fosse (1912). *Compt. Rend. Acad. Sci.* **154**, 1819.
Gerngross and Bach (1923). *Biochem. Z.* **143**, 542.
Gortner (1916). *J. Biol. Chem.* **26**, 177.
Hammarsten and Hedin (1924). Textbook of physiological chemistry.
Hardy (1905). *J. Physiol.* **33**, 351.
Hart (1901). *Z. physiol. Chem.* **23**, 347.
Jordan-Lloyd (1916). *Proc. Roy. Soc. Lond.* B **89**, 277.
—— (1920). *Biochem. J.* **14**, 147.
—— and Mayes (1922). *Proc. Roy. Soc. Lond.* B **93**, 69.
Kaye and Jordan-Lloyd (1924, 1). *Proc. Roy. Soc. Lond.* B **96**, 293.
—— —— (1924, 2). *Biochem. J.* **18**, 1043.
Kernot and Knaggs (1928, 1). *J. Soc. Chem. Ind.* **47**, 967.
—— —— (1928, 2). *Biochem. J.* **22**, 528.
—— —— (1929). *Proc. Roy. Soc. Lond.* B **105**, 280.
Knaggs (1923). *Biochem. J.* **17**, 488.
—— Manning and Schryver (1923). *Biochem. J.* **17**, 473.
—— and Schryver (1924, 1). *Biochem. J.* **18**, 1079.
—— —— (1924, 2). *Biochem. J.* **18**, 1095.
—— —— (1924, 3). *Biochem. J.* **18**, 1102.
Kossel and Kutscher (1898). *Z. physiol. Chem.* **25**, 551.
Kubelka (1918). *Kolloid Z.* **23**, 58.
—— and Taussig (1926). *Koll. Chem. Beihefte*, **22**, 150.
Kühne and Ewald (1877). *Verhandl. naturhist. med. Verein.* (Heidelberg), N.F. **15**, 198.
Kutscher (1898). *Z. physiol. Chem.* **25**, 195.
Laqueur and Sackur (1902). *Beitr. chem. Physiol. Path.* **3**, 193.
Leuchs and Manasse (1907). *Ber. deutsch. chem. Ges.* **40**, 3235.
—— and La Forge (1908). *Ber. deutsch. chem. Ges.* **41**, 2586.
Levene, Bass, Steiger and Bencowitz (1927). *J. Biol. Chem.* **72**, 815.
Lock and Thomas (1913). *Z. physiol. Chem.* **87**, 74.
Loeb (1920–1921). *J. Gen. Physiol.* **3**, 557, 667, 691, 827.
Marriott (1921). *J. Soc. Leather Trades Chem.* **5**, 280.
Northrop (1921, 1). *J. Gen. Physiol.* **3**, 715.
—— (1921, 2). *J. Gen. Physiol.* **4**, 57.

Osborne and Harris (1903). *J. Amer. Chem. Soc.* **25**, 323.
Pauli (1922). The colloid chemistry of the proteins.
Plimmer (1912). Chemical constitution of the proteins, Pt II. (Longmans.)
Porter (1921). *J. Soc. Leather Trades Chem.* **5**, 259.
—— (1922). *J. Soc. Leather Trades Chem.* **6**, 83.
Procter (1917). *First report on colloid chemistry*, 5.
—— and Wilson (1923). *Fifth report on colloid chemistry*, 13.
Reich-Herzberge (1901). *Z. physiol. Chem.* **34**, 119.
Richards and Gies (1902). *Amer. J. Physiol.* **7**, 93.
Robertson (1920). The physical chemistry of the proteins.
Rohm and Haas Co (1922). *J. Amer. Leather Chem. Assoc.* 542.
—— —— (1923). *J. Amer. Leather Chem. Assoc.* 516.
Sadikoff (1903). *Z. physiol. Chem.* **39**, 398.
—— (1904). *Z. physiol. Chem.* **41**, 15.
Schroeder (1903). *Z. physikal. Chem.* **45**, 75.
Smith (1921). *J. Amer. Chem. Soc.* **43**, 1350.
Thomas and Kelly (1925). *J. Amer. Chem. Soc.* **47**, 833.
Tolman and Stearn (1918). *J. Amer. Chem. Soc.* **40**, 264.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.
—— and Birchard (1913). *J. Biol. Chem.* **16**, 539.
Wilson (1920). *J. Soc. Leather Trades Chem.* **4**, 68, 291.
—— and Kern (1923). *J. Amer. Chem. Soc.* **45**, 3139.